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The Role of Benthic Nitrogen Fixation as a Source of New Nitrogen to the New River Estuary, NC

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The Role of Benthic Nitrogen Fixation as a Source of new Nitrogen to the New River Estuary, NC

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This thesis is submitted in partial fulfillment of

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ABSTRACT

Nitrogen (N) has been shown to limit primary production in many estuarine systems, including the New River Estuary (NRE), NC, a moderately eutrophied system with large areas of photic sediment. The NRE receives major inputs of allochthonous nutrients from agriculture and confined animal feeding operations as well as other sources. Autochthonous sources of N in the NRE include both remineralization and N-fixation. Whereas allochthonous sources are usually most important in winter/spring and during periods of high fresh water discharge, autochthonous sources are likely to become more important in summer. N-fixation, which can be performed by both autotrophic cyanobacteria and heterotrophic bacteria, was shown to vary in response to light levels, organic matter and temperature. To assess the importance of N-fixation at a system-wide scale we sampled seasonally along the estuarine gradient at multiple water depths (with a range of light availabilities) and multiple sediment depths. Benthic N-fixation activity was determined using the acetylene reduction method. Molecular characterization of the microbial communities along with the molybdate inhibition technique were used to verify the relative importance of autotrophic to heterotrophic sulfate reducing N-fixers. The majority of benthic NFix in the NRE was performed by sulfate-reducing bacteria. Although highest rates of benthic N-fixation were in the top 0 – 1 cm, a substantial portion occurred down to 10 cm in shallow and deep water samples. N-fixation rates were highest in the mid and lower estuarine sites during summer, with estimates as high as 2407 μmol N m^{-2} d^{-1}. Benthic N-fixation rates varied seasonally and were a significant source of autochthonous N to the NRE, contributing up to 19% of total new N inputs during spring.

The Role of Benthic Nitrogen Fixation as a Source of new Nitrogen to the New River Estuary, NC

CHAPTER 1

Spatial and temporal patterns of benthic nitrogen fixation in the New River Estuary, NC

Introduction

Shallow estuaries

Shallow coastal bays and estuaries are common features on the East and Gulf coasts of the U.S. and constitute at least 13% of the world's coastlines (Boynton 1996, Nixon 1982). These shallow systems provide important societal and ecosystem functions including human recreational activities and commercial fisheries production (Costanza et al. 1997). Compared to deeper coastal systems, light reaching the sediment surface in shallow systems (typically 2 to 5 m) can support the growth of a diverse community of benthic organisms including macroalgae, benthic microalgae (BMA), phototrophic bacteria, and seagrasses (Nixon 2001). Due to the large extent of the photic zone in these systems, estuaries and shallow bays can serve as a filter, retaining, transforming, and removing incoming land-derived nutrients before they pass to coastal ocean waters (Joye and Anderson 2008, McGlathery et al 2007, An and Joye 2001).

Shallow systems differ from deep systems in that primary production does not respond in a predictable or clear linear fashion to nitrogen inputs, even after adjusting for water residence time (Nixon et al. 2001), though total system production does increase with very low rates of nitrogen input. Much of the primary production in shallow systems may occur in the benthos, carried out by seagrasses, macroalgae, and BMA as opposed to phytoplankton in the pelagic zone (Nixon et al. 2001, McGlathery et al 2001, Valiela et al. 1997). The response of shallow systems to increased nitrogen loading is

strongly influenced by the benthic autotrophic community, which plays an important role in taking up, transforming, retaining or even producing water column nitrogen (N) (McGlathery et al. 2004, Nixon et al. 2001, Fulweiler et al. 2007).

Increased nitrogen loading to coastal systems can lead to a shift in the relative dominance of benthic to pelagic autotrophs due to physiological differences and competition for resources (Valiela et al. 1997). Valiela et al. (1997) proposed a conceptual model in which they hypothesized that sea grasses will dominate systems with low nitrogen loading or very short water residence times. With moderate increases in nitrogen loading or slightly longer residence times, seagrass beds and perennial macroalgae may be replaced by free-living ephemeral macroalgae. Valiela (1997) further hypothesized that phytoplankton will eventually dominate production if water residence time is sufficiently long and nitrogen concentration is high. This shift may occur because phytoplankton and macroalgae are nitrogen limited and more efficiently take up nutrients as concentrations increase; thus, increased nitrogen availability promotes algal growth, and as a result seagrass is lost as a secondary effect due to shading (Valiela et al. 1997, Duarte 1995). Seagrass production, which is affected by light availability, has also been shown to be nutrient limited in temperate ecosystems (Taylor et al. 1995, Tyler et al. 2003) despite their ability to attain nutrients via their roots, and store large amounts of nitrogen in their stems, leaves, and

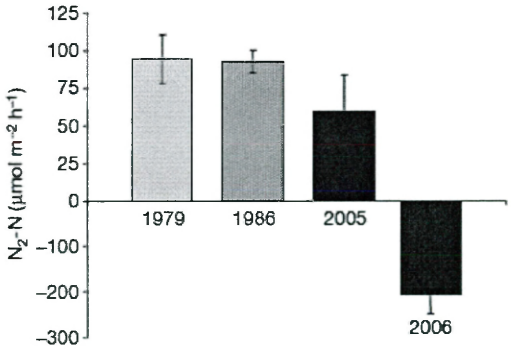


Figure 1: Mean summer N_2 fluxes in mid-bay, Narragansett Bay (Fulweiler et al. 2007). Positive rates indicate denitrification, negative rates indicate NFix.

rhizomes (Valiela 1997). BMA are expected to dominate in the absence of seagrasses until shading by macroalgae or phytoplankton limits light for BMA production (Dalsgaard 2003). The dominance of phytoplankton production will lead to increased export of nitrogen and organic matter to the coastal ocean waters (McGlathery et al., 2001, 2007).

Whereas in recent decades up to one-third of coastal areas have seen dramatic increases in nitrogen loading (National Research Council 2000) leading to increases in primary production, harmful algal blooms and eutrophication (Howarth et al. 1996, 2011, Howarth and Paerl 2008), other estuaries such as Narragansett Bay have undergone oligotrophication due in part to shifting climate and management actions. In Narragansett Bay loss of the winter-spring diatom bloom in most years between the 1970s and 1990s decreased the amount of organic matter deposited in the benthos. Fulweiler et al. (2007) and Fulweiler and Nixon (2009) reported that due to decreased organic enrichment, marine sediments in Narragansett Bay switched from being a net source of N_2 and sink of NH_4^+ to a sink of N_2 and source of NH_4^+ due to increased nitrogen fixation (NFix) and decreased denitrification (Figure 1). In a recent study in Waquoit Bay, a eutrophied estuary in Cape Cod, MA, sediments displayed net nitrogen fixation in late spring and summer with rates as high as 770 umol m^{-2} h^{-1}, as they switched from being a net N_2 source to a N_2 sink (Rao and Charette, 2012).

Sources and Sinks of Nitrogen to Coastal Ecosystems

In estuarine environments, allochthonous sources of new N include agricultural, forest, and urban runoff, confined animal feeding operations (CAFOs), sewage and

industrial waste and atmospheric deposition. Estuaries may also receive nitrogen inputs from coastal ocean waters (Howarth et al. 2011). Autochthonous sources of nitrogen include NFix, the only autochthonous source of "new" N and ammonification, the production of ammonium by microorganisms, which includes N that is fixed or remineralized, and also produced by dissimilatory nitrate reduction to ammonium (DNRA). Nitrogen deficiencies are frequently the main factor limiting primary productivity in estuarine and coastal marine environments (Paerl 2009).

In addition to "new" nitrogen, N can also be supplied by microbial remineralization of dissolved or particulate organic matter either in the water column or in sediments (Joye and Anderson, 2008; Carpenter and Capone 2008, Herbert 1999). Benthic microbial communities are a major site of remineralization and may be able to alleviate any inequality that exists between supply and removal of biologically available nitrogen necessary to sustain primary production (Herbert 1975 & 1999). N that is remineralized in the sediment can be sequestered by burial in sediments, retention in the bacterial pool, removal by coupled nitrification – denitrification and anaerobic ammonium oxidation (ANAMMOX), or transferral to higher trophic levels (Piehler and Smyth 2011, Hardison et al 2010, 2011; An and Joye 2001).

Nitrogen Fixation

Nitrogen fixation (NFix) is the natural process by which N_2 is converted to biologically available NH_3. By using the enzyme nitrogenase, nitrogen-fixing organisms reduce atmospheric dinitrogen to two moles of ammonia at the metabolic cost of 16 moles of ATP (Raymond 2004, Postgate 1982).

$$N_2 + 8\ H^+ + 8\ e^- + \underline{16}\ ATP \rightarrow\ 2NH_3 + H_2 + 16\ ADP + 16\ P_i$$

The energy required for this reaction is derived from the respiration of photosynthetically or chemosynthetically produced organic matter (Capone 1988).

The nitrogenase enzyme complex is made up of two proteins; the molybdenum-iron (MoFe) protein, which is encoded by the *nifD* and *nifK* genes, and the dinitrogen reductase (Fe) protein encoded by the *nifH* gene (Zehr and McReynolds 1989, Howard and Rees 2000). The availability of these metals in an ecosystem may regulate rates of NFix (Paerl et al. 1994, Vitousek 2002, Carpenter and Capone 2008). Hydrogen (H^+) is also reduced by the Fe only subunit of the nitrogenase enzyme alongside N_2, though the stoichiometric ratio can vary and affect results of acetylene reduction measurements when converting from C_2H_2 to N_2 (Postgate 1988, Rees and Howard 2000).

NFix plays a key role in facilitating primary production when available fixed nitrogen sources have been depleted (Bertics et al. 2010, Herbert 1999, Fulweiler et al. 2008, Raymond 2004, Zehr et al. 2003). Microorganisms that fix nitrogen (diazotrophs) include numerous taxonomic groups (Figure 2), including oxygenic photoautotrophs (heterocystous, nonheterocystous and unicellular cyanobacteria), phototrophic bacteria other than cyanobacteria (such as *Chlorobium*), aerobic and anaerobic heterotrophs (sulfate-reducing bacteria like *Desulfovibrio*), Fe oxidizers (*Thiobacillus)*, and archael methanogens (Zehr and Paerl 2008). Diazotrophic cyanobacteria are found in the open ocean, brackish and fresh water, coral reefs, and benthic environments including microbial mats (Steppe and Paerl 2005, Zehr et al. 1995, 2001, Lesser et al. 2004). Biogeochemical modeling estimates show that *Trichodesmium*, a nonheterocystous

filamentous cyanobacteria that forms colonies or aggregates found in tropical and subtropical oceans, may fix up to 25 teragrams N_2 yr^{-1} globally (Monteiro et al. 2010). Unicellular cyanobacteria, generally distinguished as representing Group A cyanobacteria (ex. *Crocosphaera)* or Group B cyanobacteria (ex. *Prochlorococcus)*, are less widespread than *Trichodesmium*, but gene surveys have shown their presence in the North Atlantic and the east subtropical Pacific Ocean, the Arabian and Mediterranean Seas (Paerl et al. 1994, Montiero et al. 2010, Zehr 2010). Proteobacteria, including sulfate reducers, that fix N have been isolated from ocean surface water as well as seagrass, salt marsh, and estuarine sediments (Burns et al. 2001, Smith et al. 2004, Kirshtein et al. 1991, Braun et al. 1999).

Sulfate reducing bacteria (SRBs), including *Desulfovibrio* spp., are important NFixers in benthic marine systems. Using the molybdate inhibition technique, Steppe and Paerl (2002) and Bertics et al (2010) showed that SRBs contribute up to 64% of nighttime NFix, up to 27% of overall activity in microbial mats, and up to 86% in bare or bioturbated shallow coastal sediments. Molybdate addition studies have indicated that SRBs contributed approximately 16% to the NFix activity in bare and eelgrass *Zostera marina* vegetated sediments (Cole and McGlathery 2012), approximately 25% of the NFix activity in the *Z. marina* rhizosphere (McGlathery et al. 1998), though other studies have shown higher contributions (70-90% for Z. *noltii,* Welsh et al. 1996; 95% for *Z. marina,* Capone 1982). Methanogenic Archaea have been found to fix N in deep sea hydrothermal vents cooperatively with sulfate reducers (Zehr 2003, Dekas et al 2009).

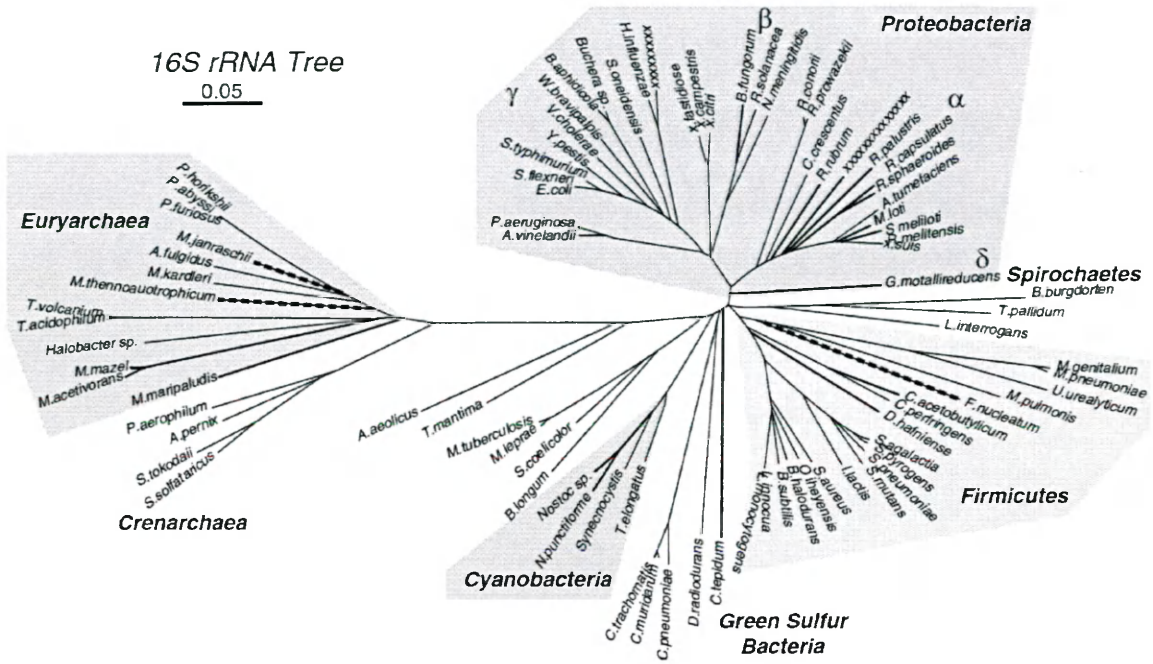


Figure 2: 16S rRNA tree indicating position of diazotrophs within major clades (from Raymond et al. 2004). The bold lines indicate diazotrophic genomes, as determined by the presence of *NifHDKEN* operons. Dashed lines indicate lineages having homologs to *NifH* and *NifD* but are not known to fix nitrogen. Shaded areas indicate NFixing lineages.

Though historically the contribution of nitrogen fixers to the marine N cycle has been underestimated, the application of modern techniques in enzymology, molecular biology, stable isotope biogeochemistry and expanding field observations have led to substantial advancements in the study of NFix in the marine environment (Mahaffey et al. 2005). Estimates of the importance of NFix to the nitrogen budget of marine ecosystems are quite varied, with global oceanic NFix in the range of $100 - 200 \times 10^{12}$ g N $year^{-1}$ (Carpenter and Capone 2008).

NFix is important to benthic macrophytes, which need high levels of fixed nitrogen to sustain primary production (Herbert 1999) with highest rates in the summer

months (Tyler et al 2003). NFix has been shown to provide up to 30% of total summer N demand for *Z. marina* vegetated sediments in the shallow coastal bays of the Delmarva Peninsula, VA (Cole and McGlathery 2012). NFix rates for these vegetated sediments were 28 times higher than bare sediments, with 90% of the fixation provided by heterotrophic epiphytic bacteria on the seagrass. Welsh et al. (1996) similarly showed that NFix contributed up to 12% of the annual nitrogen requirement of the seagrass *Zostera noltii* in the seagrass meadows of the Bassin d'Arcachon, south-west France, with average rates of 4,416 and 2,400 umol N m^{-2} day^{-1} for light and dark incubations respectively. NFix rates measured in lagoon sediments in Tekhau Lagoon, French Polynesia averaged 140 umol N m^{-2} day^{-1}, which accounted for approximately 24% of N demand by benthic primary producers (Charpy-Roubaud et al. 2001). Fulweiler et al. (2007) observed rates of 600 – 15,600 umol N m^{-2} day^{-1} in sub-tidal sediments collected from mid Narragansett Bay, USA in summer 2006, some of the highest rates measured for a marine system. NFix was found to contribute 10% of the biologically available autochthonous N, which included remineralized N, to the N budget of Hog Island Bay on the Delmarva Peninsula, VA (Anderson et al. 2010).

Primary production in mature salt marshes on the Gulf and Atlantic coasts of the US are often limited by N availability (Valiela 1983, Tyler et al 2003). Tyler et al. (2003) showed high rates of NFix (2.01± .023 mg N m^{-2} h^{-1}) in *Spartina alterniflora*-vegetated sediment (excluding aboveground growth) in the Delmarva Peninsula, VA; with 75% of NFix attributed to heterotrophic NFix. Organic matter (OM) availability, which can limit heterotrophic NFix, was shown to have an inverse relationship with NFix; Tyler et al. determined that OM availability was not limiting in this marsh

ecosystem possibly due to exudate from actively photosynthesizing plants relieving this limitation and determining seasonal patterns (2003). Epiphytic NFix rates can be as high as 2.3-3.8 g N m^{-2} y^{-1} in some Atlantic Coast salt marshes (Currin and Paerl 1998) with cyanobacteria, diatoms, green algae, and photosynthetic and heterotrophic bacteria composing the epiphytic community on standing dead *S. alterniflora* in North Carolina (Currin and Paerl 1998).

Regulation of NFix by Environmental Factors

Sediment NFix has been shown to be influenced by many physical and biological factors. Oxygen inhibition of nitrogenase, a key enzyme responsible for NFix, (Fay 1992) has been shown to be an important factor controlling NFix in N depleted coastal marine waters (Paulsen et al. 1991). Nitrogen fixing cyanobacteria, which are thought to be important NFixers in photic systems, have developed numerous strategies for protection of nitrogenase from oxygen produced during photosynthesis, including segregation of active nitrogenase into specialized cells called diazocytes (El-Shehawy et al. 2003), development of heterocysts, specialized cells for NFix that contain a protective wall and have only photosystem I (Schmetterer 1994), and by creation of a temporal window for NFix by down regulating oxygenic photosynthesis during mid-day (Staal et al. 2003). Phototrophic diazotrophs, thus, are strongly affected by light availability which is itself affected by turbulence, depth, concentrations of dissolved and particulate organic matter and particle density (Howarth et al. 1995). In addition, based on bioassays of *Trichodesmium* sp., iron has been shown to limit NFix in oceanic and coastal waters (Paerl et al. 1994, Rueter 1988).

Diel fluctuations in NFix, are affected by light availability since some organisms only fix N in the light, and others only in the dark and are determined by the local microbial community composition (O'Neil and Capone 1989). Currin et al. (1996) found that *Spartina* sediments dominated by filamentous, nonheterocystous cyanobacterial species exhibited a nighttime peak in NFix while sediments dominated by heterocystous and coccoid cyanobacterial species exhibited a daytime peak. Rates of NFix in *Salicornia virginica* vegetated sediments were lower during the day (7.7 ± 1.2 μmol C_2H_4 m^{-2} h^{-1}) and averaged 13 ± 6.6 μmol C_2H_4 m^{-2} h^{-1} at night, with highest rates observed in samples with visible cyanobacterial mats. In sediments containing *S. foliosa*, the opposite diel pattern was found; average daytime and nighttime rates of NFix were measured as 62 ± 23 and 21 ± 15 μmol C_2H_4 m^{-2} h^{-1}, respectively (Moseman 2007).

OM can limit both NFix and denitrification; thus increased OM loading can lead to increased sediment N-fix rates but once a threshold is reached, denitrification may dominate the sediment making it a net sink of biologically available N and source of N_2 (Fulweiler et al. 2007). Fulweiler et al (2007) showed that monthly additions of organic carbon (6.25 g m^{-2}) to mesocosms of sediment collected from Narragansett Bay, USA lowered rates of NFix compared to un-amended mesocosms, with additions of 12.5 g organic C m^{-2} completely inhibiting NFix (Fulweiler et al. 2008). In situ NFix rates in Narragansett Bay also varied seasonally in response to organic matter deposition, with highest rates occurring in June and August 2006.

Other factors that can influence sediment NFix include temperature, inorganic nitrogen, and salinity (Howarth 1988, Paerl 1990). The presence of ammonium has been

presumed to inhibit NFix because less energy is required to assimilate ammonium than is required to synthesize nitrogenase and fix nitrogen (Howarth 1988). Capone and Carpenter (1982) showed that NFix rates could be stimulated in salt marsh sediments by the removal of interstitial ammonium (Capone 1988). However, pore water ammonium with *in situ* concentrations of up to 650 uM, had no negative effect on NFix in sediment cores containing live eelgrass collected from the Limfjord, Denmark (McGlathery et al. 1998). NFix may also be inhibited in sediments by high concentrations of sulfate, which blocks molybdenum uptake and may inhibit growth of some diazotrophs (Howarth and Cole 1985; Marino et al 2003).

NFix Rate Measurements

The three most common techniques used for measuring NFix rates are the acetylene reduction assay (ARA), which measures the activity of nitrogenase, use of ^{15}N as a tracer to measure uptake of N_2 into sediment as reduced nitrogen ($^{15}NH_x$) and membrane inlet mass spectrometry (MIMS), which measures net uptake (NFix) or release of N_2 (denitrification, DNF) relative to argon. ARA, first described by Stewart et al. (1967), uses the reduction of the triple bonded acetylene (C_2H_2) to ethylene (C_2H_4) as a proxy indicator of the reduction of dinitrogen to ammonia. A theoretical ratio of three moles of acetylene reduced to one mole of N_2 reduced to NH_3 is based on the number of reducing equivalents needed to reduce C_2H_2 to C_2H_4 relative to the number required by nitrogenase to reduce N_2 to NH_3 (Zehr and Montoya 2007, Montoya et al. 1996). A more realistic ratio of 4:1 has also been proposed, based on the stoichiometry for the nitrogenase reaction: $8H^+ + 8e^-_. + N_2 \rightarrow 2NH_3 + H_2$ (Staal 2001, Zehr and Montoya 2007,

Mulholland et al. 2004, Montoya et al. 1996). An overestimation of NFix rates may result from using the 3:1 ratio due to C_2H_2 inhibition of hydrogenase, which restores reducing equivalents to nitrogenase, thereby resulting in a greater yield of C_2H_4 (Mulholland et al 2004, Postgate 1988, Seitzinger and Garber 1987). Verification of the 4:1 ratio has been provided by comparing acetylene reduction to $^{15}N_2$ uptake and also by use of membrane inlet mass spectrometry (MIMS). The 4:1 ratio was shown to be accurate for *Trichodesmium* sp. using acetylene reduction and $^{15}N_2$ uptake (Mulholland et al. 2004) and *Anabaena variabilis* using acetylene reduction and MIMS (Jensen and Cox, 1983).

^{15}N tracer amendment experiments are advantageous in that they directly measure the process of NFix but they are time-consuming and much more expensive to analyze than ARA. Additionally, ARA assays can be repeatedly sampled over time, which is not possible with ^{15}N enrichments, in addition, ARA assays are easily and cost-effectively analyzed by gas chromatography (Seitzinger and Garber, 1987). The sensitivity of ^{15}N enrichments is several orders of magnitude less than ARA (Capone 1988) and requires much longer incubation times to detect low rates of NFix. A comparison of the ^{15}N tracer method to ARA done by Šantrůc̆ková et al. (2010) for marsh sediment and cyanobacterial mats; showed a N_2:C_2H_4 range of 1:0.60 to 1:4.31, respectively.

Use of MIMS to estimate net NFix has been described by Fulweiler 2007; Gardner et al. 2006 and McCarthy et al. 2007. Whereas MIMS is a direct measurement of N_2 consumption and requires no conversion factor as ARA does, a limitation of MIMS is that it estimates only net consumption of N_2 and may be confounded by concurrent production of N_2 by DNF or anammox

Molybdate Inhibition

Molybdate inhibition, as first described by Oremland and Capone (1988), involves the use of sodium molybdate to inhibit sulfate reduction in order to determine the extent to which sulfate-reducing bacteria are responsible for patterns of NFix (McGlathery 1998). Molybdate, a specific inhibitor of sulfate-reducing bacteria uncouples energy production from NFix and rapidly depletes ATP pools (Oremland and Capone 1988, Taylor and Oremland 1979). In this method, molybdate is added as Na_2MoO_4 to sediment samples and ARA is measured concurrently in amended and un-amended samples (Bertics 2010, McGlathery et al. 1998).

Identification and Quantification of N fixing Bacteria

Many studies have recently been done to determine the identity and diversity of benthic N_2 fixers in multiple habitats using the polymerase chain reaction (PCR). As a result the nitrogenase gene database (*nifH* gene) has become one of the largest non-ribosomal gene datasets for uncultivated microorganisms (Zehr et al. 2003). The majority of phylogenic analysis done thus far to determine nitrogenase diversity has been based on analysis of *nifH*, although *nifD* and *nifK* sequences are being used to differentiate between closely related strains (Zehr and McReynolds 1989).

NifH sequences obtained using PCR amplification from highly reduced sediments of anoxic mud taken in the mid-portion of the Chesapeake Bay clustered closely with each other and with known anaerobic organisms (Burns 2002). Two of the ten sequences clustered with *Desulfobacter curvatus*. The authors compared sediments taken from the Chesapeake Bay with sandy surficial sediments taken from the Neuse River, NC. The

sequences taken from the Neuse River, NC sediments associated with previously reported sequences from marine mats and the *Spartina* rhizosphere and were distant from those taken from the Chesapeake Bay (Burns 2002). Specific environmental site characteristics seemed to select for different diazotroph communities. Whereas the mesohaline Chesapeake Bay site was organic rich and dominated by sulfate reducing NFixers, the oligohaline Neuse River site was relatively low in total organic matter and included NFixers typically found in microbial mats and *Spartina* rhizospheres (Burns 2002, Marvin-DiPasquale and Capone 1998, Christian et al. 1991). The presence of NFix genes identified through PCR does not necessarily mean that NFix is actively occurring in these environments, only that there is the potential to produce the enzyme for NFix.

For a 3-year period, Steppe and Paerl (2005) measured NFix and diazotroph community composition in sand flat and intertidal microbial mats located in an estuarine reserve near Beaufort, NC. Most of the *nifH* sequences obtained using PCR from these sites clustered in the obligately anaerobic deltaproteobacterial sulfate reducing group, though representative sequences from cyanobacterial and the beta and gamma proteobacterial groups were also present (2005). The few cyanobacterial sequences analyzed were similar to the *Lyngbya lagerheimmi nifH* sequence.

Terminal restriction fragment length polymorphism (T-RFLP) is a method used for profiling microbial communities based on PCR amplification of a target gene with one or both the primers having their 5' end labeled with a fluorescent molecule. PCR amplified target genes are digested with restriction enzymes to produce fluorescently labeled terminal restriction enzyme fragments, which are then read using a DNA sequencer. Using T-RFLP, Bertics et al. (2010) observed cyanobacterial *nifH* genes

within phototrophic microbial mat samples taken in an intertidal lagoon located in Catalina Harbor, Catalina Island, California, that exhibited high rates of NFix. SRB *nifH* genes (including those from *Desulfovibrio spp.* and *Desulfobacter spp.*) were found in surface samples as well as in deeper sediment samples where NFix was occurring (2010).

Real-time quantitative polymerase chain reaction (RT-qPCR) is used to amplify and simultaneously quantify a targeted RNA or DNA molecule as an absolute number of gene copies normalized to DNA input or additional normalizing genes. The use of short oligonucleotides as probes allows specific detection and quantification of related phylotypes even at the species level (Zhang et al. 2007). Microarrays and macroarrays developed to target specific functional groups, including *nifH*, allow for rapid characterization of diverse communities with quantitative comparisons and high-throughput [Church et al. 2005, Gibson 2002]. Reverse transcriptase PCR (RT-PCR) of RNA molecules makes it possible to assay for cells that are actively expressing specific genes at the time of sampling (Zani et al. 2000). By comparing the sequences obtained by PCR with those obtained by RT-PCR it is possible to investigate the diversity of organisms expressing genes in different habitats and under different environmental conditions (Kowalchuk et al. 1999).

Objectives/Hypotheses

The overarching objective of this study was to examine the spatial and temporal distributions and regulation of benthic NFix, an autochthonous source of new nitrogen, in the New River Estuary (NRE), North Carolina. By scaling rates of benthic NFix measured at the m^2 level to the entire estuarine system we were able to determine the

seasonal contribution of NFix to total nitrogen inputs to the system. Molecular genetic studies were also conducted to determine the microbial community fixing nitrogen and changes in its composition down the estuarine gradient. This study was part of a larger comprehensive study, the Defense Coastal Estuarine Research Program (DCERP), with the objective of distinguishing the effects of Marine Corps Base Camp Lejeune (MCBCL) activities from those of the surrounding area on estuarine processes.

- **Objective 1. Examine the spatial and temporal distribution of benthic NFix activity and its variation with environmental factors.**
  - *Benthic NFix will increase as a function of water column temperature and will be highest at estuarine sites that have lowest light attenuation, lowest sediment NH_4^+ and organic enrichments.*
  - *Benthic NFix will decrease with increasing sediment and water column depth.*

- **Objective 2. Examine the contribution, distribution, and relative abundance of cyanobacteria and sulfate reducing bacteria (SRBs) in relation to benthic NFix rates.**
  - *Phototrophic diazotrophs at the sediment surface are responsible for the majority of benthic NFix at shallow water depths.*
  - *SRBs will contribute a significant amount to benthic NFix throughout the estuary.*
  - *SRBs will comprise a larger portion of the microbial nitrogen fixing population in non-photic sediments.*

- **Objective 3. Examine the estuarine-wide role of benthic NFix as a source of new nitrogen compared to total autochthonous and allochthonous sources in the NRE.**
  - ✧ *Benthic NFix displays strong seasonal variation and is an important estuarine-wide source of new N in the NRE especially during summer months.*

Methods

Site Description

The New River Estuary (NRE) (Figure 3) is located in southeastern North Carolina, and is mostly surrounded by Marine Corps Base Camp Lejeune (MCBCL) with the city of Jacksonville located at the head of the estuary. The NRE is characterized as a shallow water body with over half the estuary having a depth of less than 2m (Figure 4). It has an estuarine surface area of approximately 88 km^2 and is fed by a watershed estimated to be 462 square miles (Tomas et al. 2007). Over half of the shoreline of the NRE is sediment bank, with 21% composed of marsh. Marshes within the MCBCL region are typically dominated by smooth cordgrass *(Spartina alterniflora)* and black needle rush (*Juncus roemerianus*). The average flushing time of the NRE is 64 d as the mouth of the estuary is a narrow inlet constrained by barrier islands; however, flushing time varies both along the estuary and in response to climatic conditions (Ensign 2004).). The tidal range of the NRE varies from approximately 43cm near the mouth of the estuary to 13cm towards the head (per Carolyn Currin comm.)

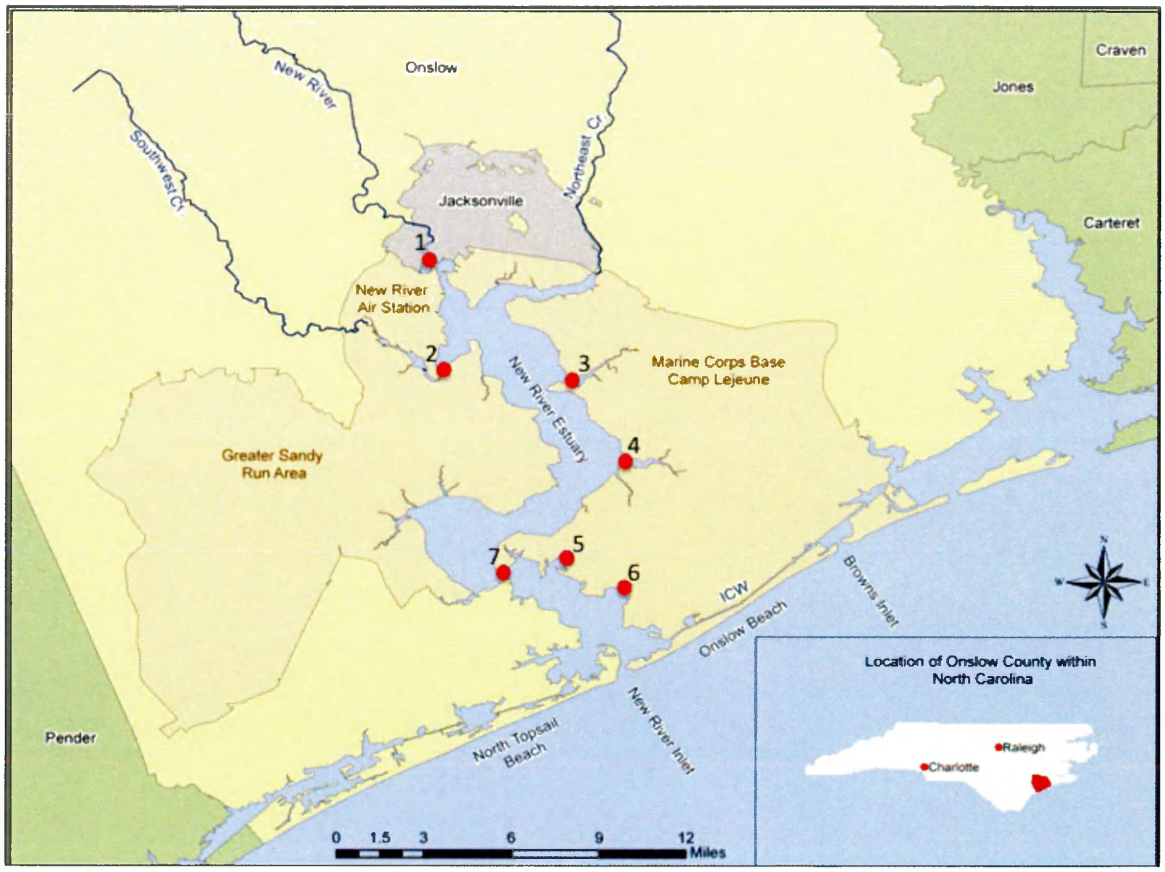


Figure 3: The New River Estuary and surrounding region.

Table 1: NRE sampling sites

Site	Location – Latitude (N)	Location – Longitude (W)
Site 1: Jacksonville (JACK)	N 34 45.073	W 077 26.125
Site 2: Southwest Creek (SWCR)	34 41.032	077 25.628
Site 3: Wallace Creek (WALL)	34 40.922	077 21.968
Site 4: French Creek (FRCR)	34 38.401	077 20.291
Site 5: Courthouse Bay (CTBY)	34 35.404	077 22.103
Site 6: Traps Bay (TRBY)	34 34.129	077 20.230
Site 7: Pollocks Point (PP)	34 34.822	077 24.102

The NRE exhibits moderate eutrophication and strong gradients of dissolved inorganic nitrogen (DIN), light attenuation, benthic and pelagic chlorophyll *a*, and chromophoric dissolved organic matter (CDOM). There is a down-estuary gradient for benthic and water column chl *a*, extractable NH_4^+, and dissolved organic and inorganic nitrogen (DIN, DON). The fact that the highest values are up-estuary suggests that on an annual basis the New River watershed is the main external source of nitrogen to the estuary (DCERP Annual Report 2009). Major allochthonous sources of N to the watersheds that drain to the NRE include agricultural runoff, runoff from confined animal feeding operations (CAFOs), forest clear-cutting, urban surfaces, atmospheric deposition, and sewage spills (DCERP AE3 2009 annual report). Current results predict that 64% of the annual allochthonous (i.e., external) total N load to the estuary originates from off-Base sources, with loads from the Base contributing only 8%, 6% from direct atmospheric deposition, 15% from Onslow Bay and 7% from the waste water treatment facilities (WWTF) (DCERP Final Report 2012).

Recent expansion of personnel on MCBCL, accompanied by new housing development, forest clear cutting, building of roads and base infrastructure, have likely led to increased sedimentation and nitrogen inputs to the estuary. These effects along with other man-made discharge events and hydrologic forcing driven by climatic changes determine nitrogen and organic matter processing and resultant water quality in the NRE.

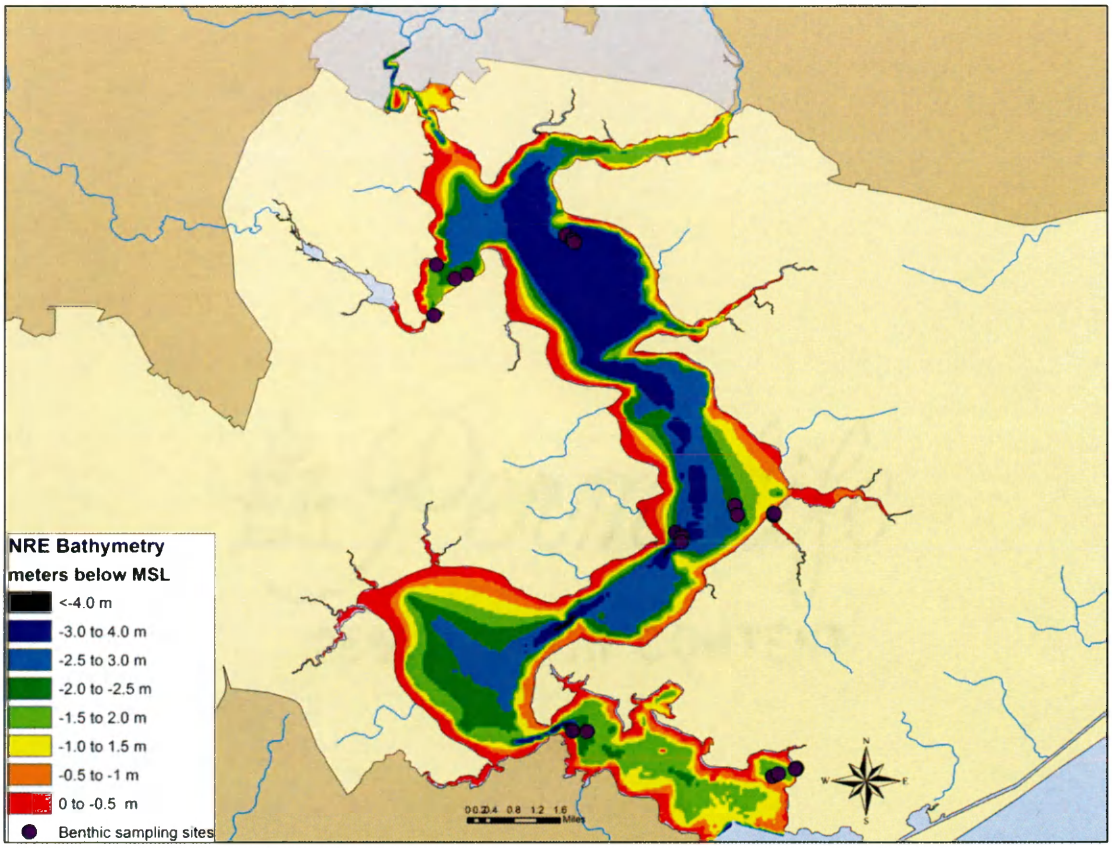


Figure 4: NRE bathymetry (mean sea level (MSL); NAVD88) and depth experiment sampling stations in the upper, middle, and lower regions of the estuary (McNinch 2009) Image courtesy of Jennifer Stanhope.

Hydrologic forcing plays an important role in the NRE, affecting N availability, which has been shown to limit primary production (DCERP 2009, 2010 Annual Report). When comparing a normal year (2009) and a high discharge year (2010), algal bloom density and frequency were highest in 2009 when high nitrogen loads were combined with moderate flushing as opposed to 2010 when increased flushing lowered the residence time of the nitrogen loads (DCERP Annual Report 2010). Freshwater discharge from the New River can reduce photic area, which ranged from 46 - 97% of total

estuarine bottom (2008-2011), due to the loads of CDOM and particulates thereby affecting benthic chl a (DCERP Final Report 2012). The nutrients delivered in freshwater discharge support phytoplankton blooms, which further increase light attenuation and reduce the photic area. Autochthonous nitrogen regenerated internally within the estuary was more important during drier summer periods when allochthonous N sources were at a minimum to support phytoplankton blooms (DCERP Annual Report 2010). A highly productive benthos, as indicated by high sediment chlorophyll, suggests that benthic processes are likely to play an important role in retaining and transforming nitrogen and reducing the impacts of nitrogen enrichment to the system.

Experimental Design

Seasonal NFix measurements

For determination of spatial and temporal variation of benthic NFix across the NRE sediment cores were collected seasonally (spring, summer, winter, and fall) from May 2009 to August 2011 at six sites (Figure 3) for determination of NFix rates (6 cores per site) and sediment characterization (3 cores per site). Sediment samples (10 cm) for NFix and sediment characterization were taken at 0.5 m mean low water (MLW) in cores that are 20 cm long with a 5.5cm diameter. There were a total of ten seasonal samplings collected at 0.5m water column depth, including those from the multiple water column depth studies described below. Sediment cores were transported in insulated water baths back to VIMS for all experiments. Upon arrival at VIMS, cores were placed in stirred site water baths with their lids removed to equilibrate over night at in situ temperatures in

an environmental chamber. Sediment characterization samples were sectioned at multiple sediment depths along with NFix cores and analyzed for sediment organic content and bulk density, sediment nutrient concentrations (NO_x and NH_4^+), and benthic chlorophyll a (Table 2). Sediment samples were dried at 50°C to constant weight (±0.01g) to determine bulk density and then combusted at 500°C for 5 hours to calculate percent organic matter. Sediment extractable nutrients were determined by methods described in Anderson et al. (1997). Benthic chlorophyll in the top 0-3mm of sediment was analyzed as described in Neubauer et al. (2000) and read on a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer) at 665, and 750 nm for determination of chlorophyll-*a*.

Estuarine-wide NFix rates

In order to scale rates measured at the m^2 scale to the entire estuary, it was essential to take depth and, thus, light availability into account across the estuary. Randomized sampling was stratified at three water column depths, based on 2009 bathymetry (McNinch; Figure 4). During July 2010 and April 2011 NFix rates were measured in triplicate at upper, mid, and lower estuary sites randomly selected from GIS maps within each depth contour. Sediment cores were collected for NFix and sediment characterization and sub-sampled at 0-1cm, 1-3, and 5-10cm depth intervals. Rates of NFix and sediment characteristics were determined by methods described below (Table 2). Estuarine wide production of NH_3 by NFix was compared to allochthonous loads of N to the NRE (DCERP Final Report 2012, Maxey M.S.Thesis 2012).

Table 2. Methods: Sediment and Site characterization

Analyses	Method/Instrument	Reference
Sediment Organic Content (%)	Loss on ignition (500°C)	
Bulk density (g dry weight/mL)	Dry @ 50°C until constant weight	Anderson et al. 1997
Sediment Nutrients ($mmol/m^2$)	Potassium chloride-extraction; Lachat auto analyzer (Lachat Instruments, Milwaukee, WI, USA)	Anderson et al. 1997; Solorzano 1969, Keeney and Nelson 1982
Benthic Chlorophyll *a* (mg/L)	Acetone Extraction/ spectrophotometry; Shimadzu UV-1601 Spectrophotometer	Neubauer et al., 2000; Lorenzen, 1967

Determinations of NFix rates

NFix rates were measured using the acetylene reduction technique with a ratio of 4:1 between the total moles of acetylene reduced to moles of N_2 reduced. Subsamples (see below) collected from sediment cores were placed in 60 ml serum bottles and acetylene, generated from the reaction of CaC_2 and deionized water, was added to occupy approximately 20% of the headspace. Blanks containing only acetylene and controls containing sediment samples without acetylene were incubated along with the rest of the samples. No production of ethylene was observed in the control treatments containing sediment without acetylene, suggesting that the production of ethylene was due solely to nitrogen fixation.

Samples were incubated for 6 hours at in situ temperature in light (500 μE) and dark (see details below). After incubation, the headspace of the incubation bottles was mixed, sub-sampled, and gas samples transferred to Hungate tubes, stored upside down in

tap water until analyzed. Ethylene concentrations were analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (220°C, oven temp 80°C). Ethylene and acetylene were separated using a 6 ft Poropack N Column with a carrier gas (ultra-pure carrier grade He) flow rate of 20 ml min^{-1}. Ethylene concentrations were determined by comparison of peak values to a three point linear regression of pure ethylene standards (10 ppm, 5 ppm, and 1 ppm) and converted to ammonia using a molar ratio of 4:1.

Variation of NFix rates in the light and dark and through the sediment profile

Sediment cores were sub-sampled at multiple sediment depths: 0-1 cm, 1-3 cm, 5-7 cm, and 8-10 cm. Surface sediments (0–1 cm) were incubated aerobically in the light at an intensity of 500 $\mu E\ m^{-2}\ s^{-1}$, determined to be a saturating irradiance from photosynthesis vs. irradiance curves performed on similar sediment samples from the NRE (Brush 2012), and anaerobically in the dark. All other sediment sections were sparged with N_2 gas to produce anaerobic conditions and incubated in the dark in an environmental chamber at in situ temperatures. Subsamples collected for the estuarine scaling studies included: 0-1 cm, 1-3 cm, and 5-10 cm. The surface sediments were incubated in the light and dark; deeper sediment sections were incubated anaerobically in the dark, as described above. Rates measured for each sub-section were integrated over 10cm and averaged by site and season.

Salt Marsh NFix

Sediment and epiphytic NFix rates were measured using the acetylene reduction technique as described above for samples collected from two salt marsh sites in the New River Estuary in July 2009; Pollocks Point and Traps Bay (Figure 3). Three sediment cores were collected from three stations positioned between the upper and lower extent of the tides at each site; elevations ranged from 0m to 0.36m (MSL) (Figure 5, Table 3). All stations were dominated by *Spartina alterniflora* except Pollocks Point station 3 where *Distichlis spicata* was the dominant macrophyte. Standing dead macrophyte stems were also collected randomly from each station. Sediment cores were sub-sectioned at 0-1 cm and 4-5 cm and sections were placed in gas tight serum bottles. Three 1-inch shoots of marsh grass were added to each of three serum bottles per station totaling nine per incubation time per site (54 total). All samples were amended with 200 uL of filtered (pre-combusted GFF) seawater (salinity, 23.7) for the first two incubations and amended with 90ml of seawater for the final incubation to simulate high-tide. In order to determine how NFix varies as a function of time of day samples from three sediment cores and three bottles containing stems were incubated for 3.5 h at three different time periods of day; 7:30 am - 11am, 1 pm - 4:30 pm, and 6:30 pm – 10 pm. The 0-1 cm samples were incubated aerobically in the light; the 4-5 cm samples were sparged with N_2 gas to create an anoxic environment prior to incubation. All samples were placed in an outdoor water bath with running seawater at the NOAA Center for Coastal Fisheries and Habitat Research, Beaufort, NC. The light samples received full ambient sunlight and the dark samples were covered with shade cloth. Gas samples were transported back to VIMS in gas-tight Hungate tubes held under water for gas chromatographic analysis of

ethylene concentrations. Blanks containing only acetylene were incubated and measured concurrently with the sediment and epiphyte samples.

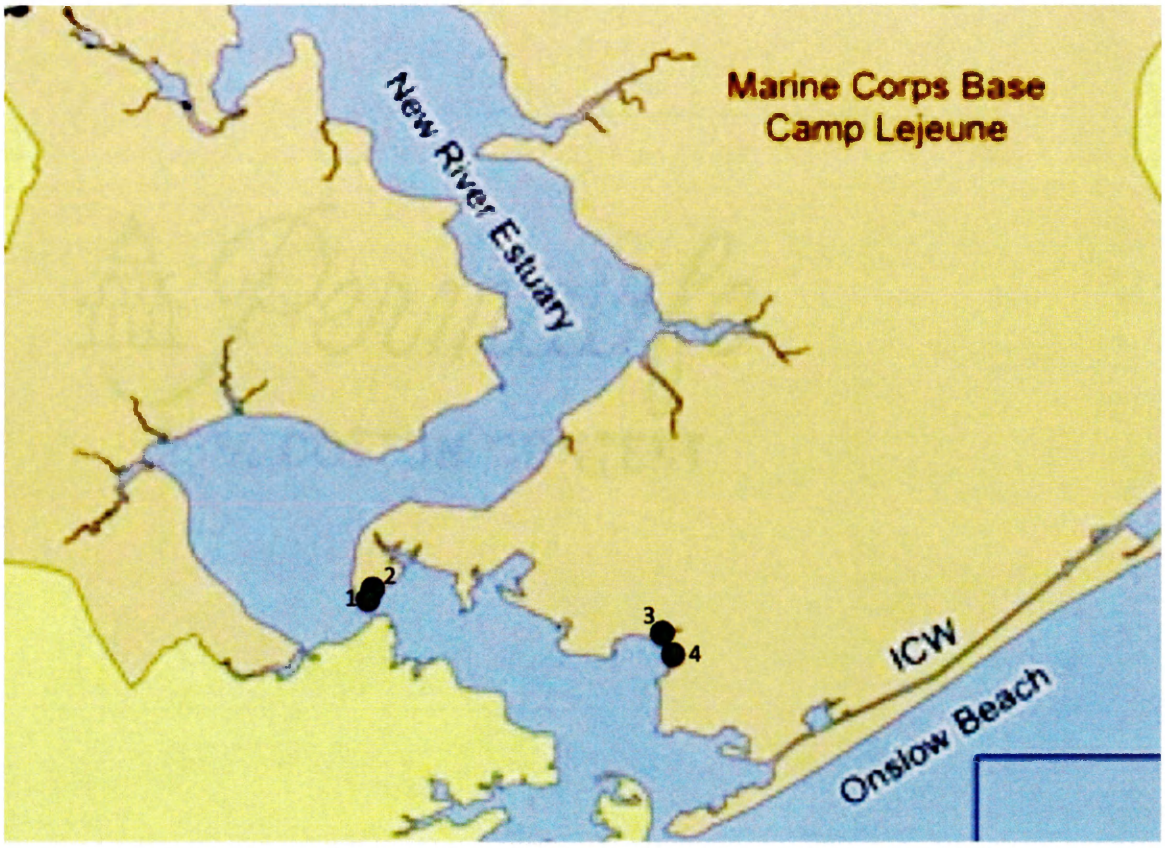


Figure 5: NRE marsh sampling sites and average site elevation (meters above mean sea level (MSL); NAVD88)

Table 3: NRE marsh sampling sites

Site	Location – Latitude (N)	Location – Longitude (W)	Elevation
1: Site A Pollocks Point - Marsh shoreline			
Station 1	34 34.822	077 24.102	0.0 m
Station 2	34 34.8252	077 24.096	0.18 m
2: Site A Pollocks Point Upper - Marsh upland			
Station 3	34 34.9494	077 24.0174	0.36 m
3: Site B Traps Bay – Creek Station 1			
Station 1	34 34.0908	0.18 m	0.01 m
4: Site B Traps Bay Bridge - Shoreline			
Station 2	34 34.0932	0.36 m	0.22 m
Station 3	34 34.3956	0.0 m	0.0 m

Statistical Analysis

Statistical analyses were performed using Statview (SAS Institute). Analysis of Variance (ANOVA) along with Tukey post hoc tests were performed to examine the effects of site, season, and water column depth on individual NFix measurements. Estuarine sediment NFix data were tested for the assumptions of ANOVA (normally distributed, equality of variance) using the Kolmogorov–Smirnov Normality Test and the F-test and Bartlett's tests for homogeneity of variance. NFix measurements were log transformed to meet the assumptions of ANOVA. One way ANOVAs were used to compare seasons within regions for each year of measurement. Two way ANOVAs were used analyze variation in sediment characteristics with site and season and also interaction effects. Differences were considered significant at $p < 0.05$. Tukey's pair-wise and multiple-comparison tests were used to determine differences between factors from significant ANOVA tests. Residual sum of squares and mean squares were calculated to

determine how much of the variance is attributable to random error and the error of the variance, respectively.

Simple linear regression analyses were used to examine correlations between mean NFix rates and environmental factors; temperature, OM%, benthic chlorophyll a, and sediment mineralization. Multiple linear regressions showed no significant improvement. Salt marsh NFix measurements did not meet the assumptions of ANOVA, therefore, nonparametric tests, including Mann-Whitney and Kruskal-Wallis tests were used to compare measurements.

Results

Sediment Characteristics

From 2009-2011, sediment characteristics, including extractable NH_4^+, organic content, and benthic chlorophyll, were assessed seasonally at six sites along the NRE estuarine gradient. Results of a two-way ANOVA (Statview), performed on these data, are shown in Table 4. Benthic chlorophyll *a* (0-3mm) (Figure 6A) data were significantly different by site and season with highest BMA biomass in the fall and at mid-estuary sites. Organic content integrated over the 10-cm sediment profile varied significantly by site and season and was always significantly higher at up-estuary sites with values decreasing along the estuary gradient (Figure 6B) (annual average 18-28% at SWCR and JACK). Sediment extractable NH_4^+ (0-10cm) (Figure 6C) data did not vary significantly by season but were significantly higher at the up estuary site, JACK. Mean annual values ranged from 4.99 (TRBY), to 6.84 (WALL), to 45.19 mmol N m^{-2} (JACK). The down-estuary gradient in organic matter content and extractable NH_4^+ suggests that the source of particulate organic matter and nutrients is at the head of the estuary.

Figure 6: New River Estuary sediment characteristics. (A) mean benthic chl a (0-3mm), (B) mean organic matter content (%) (0-10cm), (C) mean sediment extractable NH_4^+ (0-10cm) (mean ± standard error) at six sites from May 2009 to September 2011.

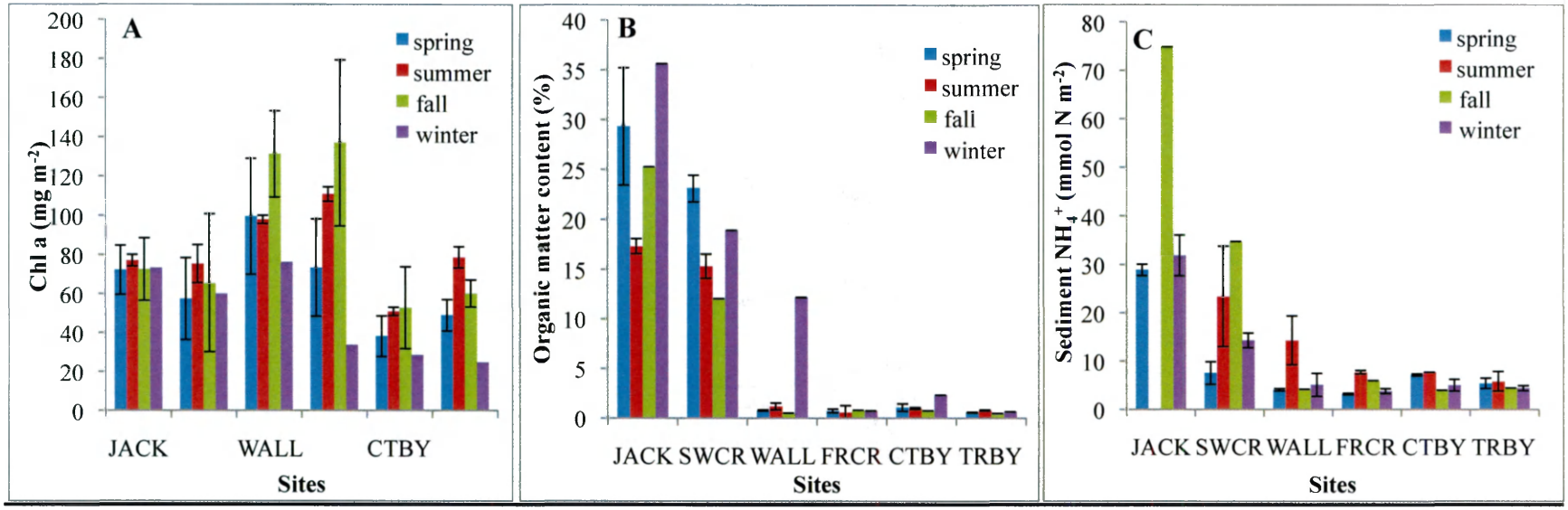


Table 4: Summary of the Two-Way ANOVA's of Benthic Chlorophyll *a* (0-mm to 3-mm Depth Horizon), Sediment Organic Matter Content (0cm to 10cm Depth Horizon), and Sediment Extractable NH_4^+ (0cm to 10cm Depth Horizon) by Site and Season Measured from May 2009 to June 2011. Significant effects are in bold; asterisks are used to show interactions.

Benthic chlorophyll a (0mm – 3mm)						
Parameter	Df	Sum sq	Mean Sq	F value	P Value	
Site	5	59752	11950	20.14	**<.0001**	(WALL>FRCR, JACK, SWCR, CTBY, TRBY) (FRCR and JACK> CTBY and TRBY)
Season	3	86828	28942	48.76	**<.0001**	(Fall > Spring and Summer > Winter)
Site * Season	15	38040	2536	4.27	**<.0001**	
Residual	117	69442	593			
Sediment organic content (%) (0 cm–10 cm)						
Parameter	Df	Sum sq	Mean Sq	F value	P Value	
Site	5	19634	3926	48.45	**<.0001**	(JACK>SWCR>FRCR, WALL, CTBY, TRBY)
Season	3	1525	508	6.27	**0.0005**	
Site * Season	15	1327	88	1.09	0.37	
Residual	161	13050	81			
Sediment extractable NH_4^+ (0 cm–10 cm)						
Parameter	Df	Sum sq	Mean Sq	F value	P Value	
Site	5	2337	467	9.29	**<.0001**	(JACK>SWCR,WALL,FRCR,CTBY,TRBY)
Season	3	354	118	2.35	0.074	
Site * Season	15	626	42	0.83	0.64	
Residual	186	93356	50			

Seasonal NFix measurements

NFix rates were estimated from sediment samples collected seasonally from 2009-2011 at 0.5m (MLW) (Figure 7). There were no significant differences between NFix measurements among sites within each of the three regions; thus, to increase the number of measurements per region, the sites within each region were combined (UP – JACK & SWCR, MID – WALL & FRCR, LOW – CRTB & TRBY). Figure 7 shows that rates varied by region and were significantly higher in warm months (Table 5). In the upper and lower regions of the estuary, season was only a significant factor in 2009, with rates highest in the summer. In the mid estuary, benthic NFix rates were significantly higher in summer 2009, and spring 2010, with no significant difference between seasons in 2011.

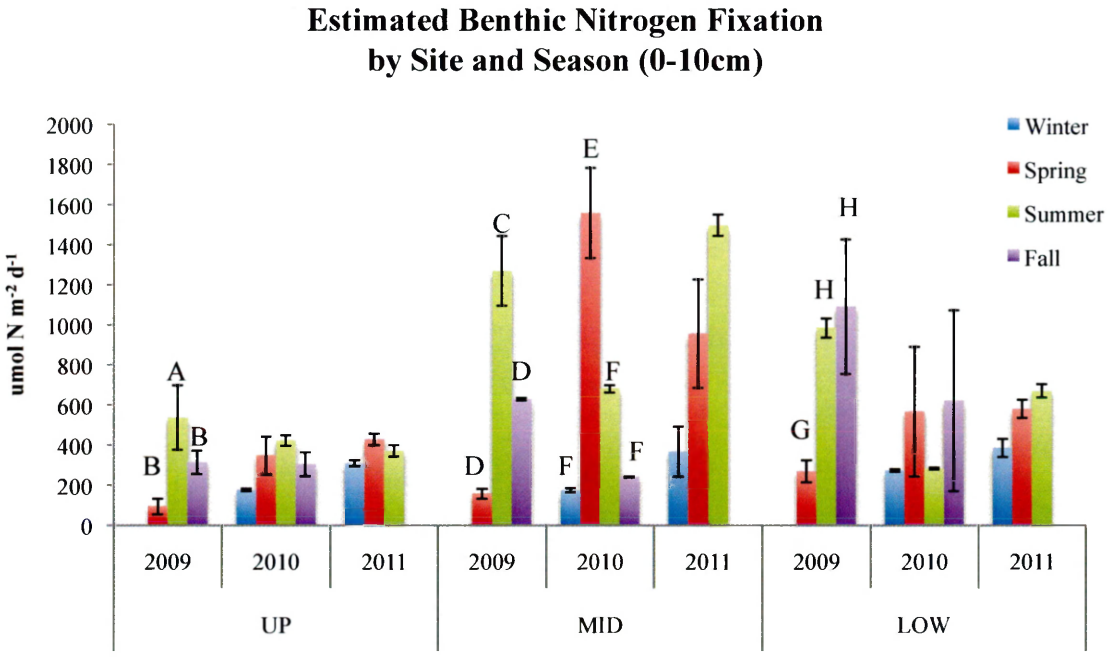


Figure 7: Estimated Daily NFix rates for 0-10 cm sediment depth, water column depth of 0.5m (2009 – 2011). Significant differences represented by difference in letter coding (ANOVA, post-hoc Tukey test (Table 4). Error bars indicate standard error.

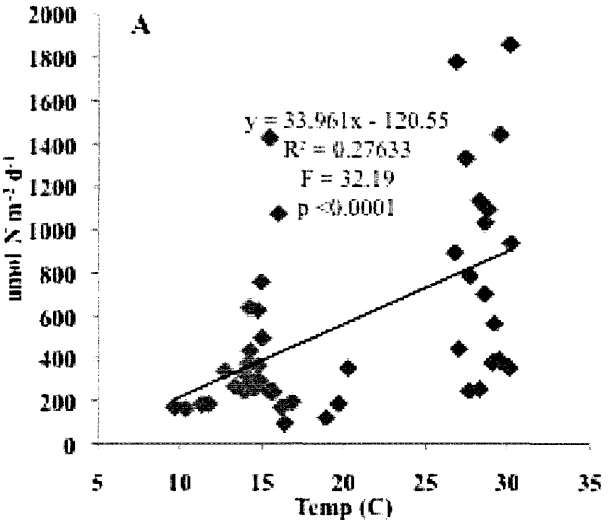
Table 5: One-Way ANOVA summary table for annual regional NFix measurements at 0.5m water column depth by season.

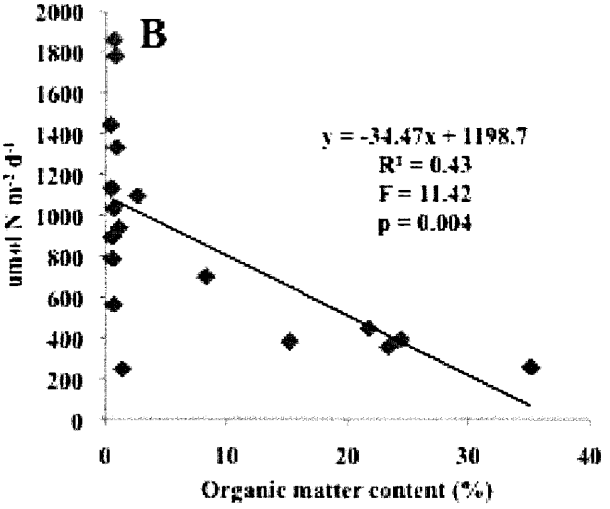
One-Way ANOVAs of NFix (0cm to 10cm Depth Horizon)						
Parameter	Df	Sum sq	Mean Sq	F value	P Value	
NFix (0-10cm) 2009 UP						
Season	2	1.794	0.897	9.581	**0.0013**	(summer and fall > spring)
Residuals	19	1.779	0.094			
NFix (0-10cm) 2010 UP						
Season	2	0.564	0.282	1.97	0.1555	
Residuals	33	4.722	0.143			
NFix (0-10cm) 2011 UP						
Season	2	0.061	0.061	0.61	0.4432	
Residuals	22	2.184	0.099			
NFix (0-10cm) 2009 MID						
Season	2	2.496	1.248	9.274	**0.0011**	(summer > fall and spring)
Residuals	23	3.095	0.135			
NFix (0-10cm) 2010 MID						
Season	2	3.667	1.834	6.441	**0.0043**	(spring > summer, fall and winter)
Residuals	22	6.123	0.278			
NFix (0-10cm) 2011 MID						
Season	2	1.439	1.439	5.172	0.0331	
Residuals	23	3.418	0.149			
NFix (0-10cm) 2009 LOW						
Season	2	2.857	1.428	9.611	**0.0009**	(summer and fall > spring)
Residuals	23	3.418	0.149			
NFix (0-10cm) 2010 LOW						
Season	2	0.255	0.127	0.529	0.5938	
Residuals	33	7.94	0.241			
NFix (0-10cm) 2011 LOW						
Season	2	0.319	0.319	2.468	0.1305	
Residuals	22	2.847	0.129			

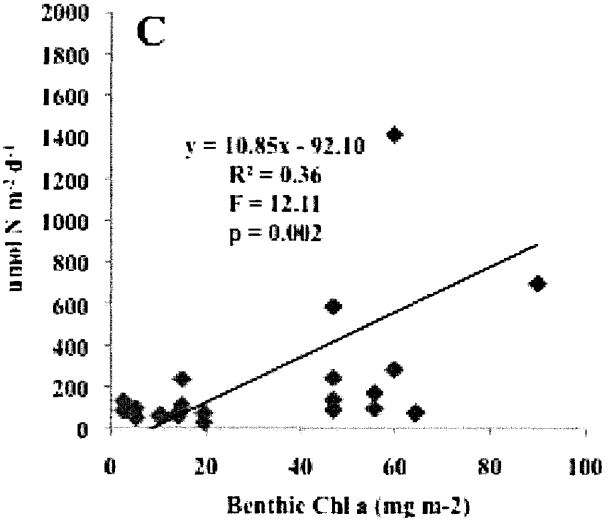
NFix rates, which varied seasonally, were significantly correlated with temperature for all dates (Figure 8A). Total NFix rates integrated over 10 cm showed a negative correlation with organic matter content, but the relationship was only significant in summer (Figure 8B). Benthic chl a concentration was negatively correlated with surface NFix in 2009 and 2010 and positively correlated in 2011, though only significant in 2011(Figure 8C).

Depth profiles of sediment NFix showed that rates decreased with sediment depth throughout the estuary (Figure 9). A two way ANOVA was used to look at the effects of sediment depth, season, and their interaction effect on NFix rates. Rates were highest in the surface 0-1cm depth (mean ± SE, 240 ± 40 umol m^2 d^{-1} over all sites and dates), which were significantly higher (Table 6) than the 1-3cm depth (112 ± 13 umol m^2 d^{-1}), the 5-7 cm depth (54 ± 5 umol m^2 d^{-1}), and the 8-10 cm depth (44 ± 4 umol m^2 d^{-1}). NFix rates through the sediment profile varied significantly with season, with highest rates in summer (Table 6). Results showed a significant interaction effect between season and sediment depth on rates measured in all sediment sections.

Figure 8. Sediment NFix 0-10cm regressed with (A) temperature (2009-2011), (B) organic matter % (summer only 2009-2011) and (C) sediment chlorophyll a (mg m^{-2}) (2011).


A
y = 33.961x - 120.55
R² = 0.27633
F = 32.19
p <0.0001
umol N m-2 d-1
Temp (C)


B
y = -34.47x + 1198.7
R² = 0.43
F = 11.42
p = 0.004
umol N m-2 d-1
Organic matter content (%)


C
y = 10.85x - 92.10
R² = 0.36
F = 12.11
p = 0.002
umol N m-2 d-1
Benthic Chl a (mg m-2)

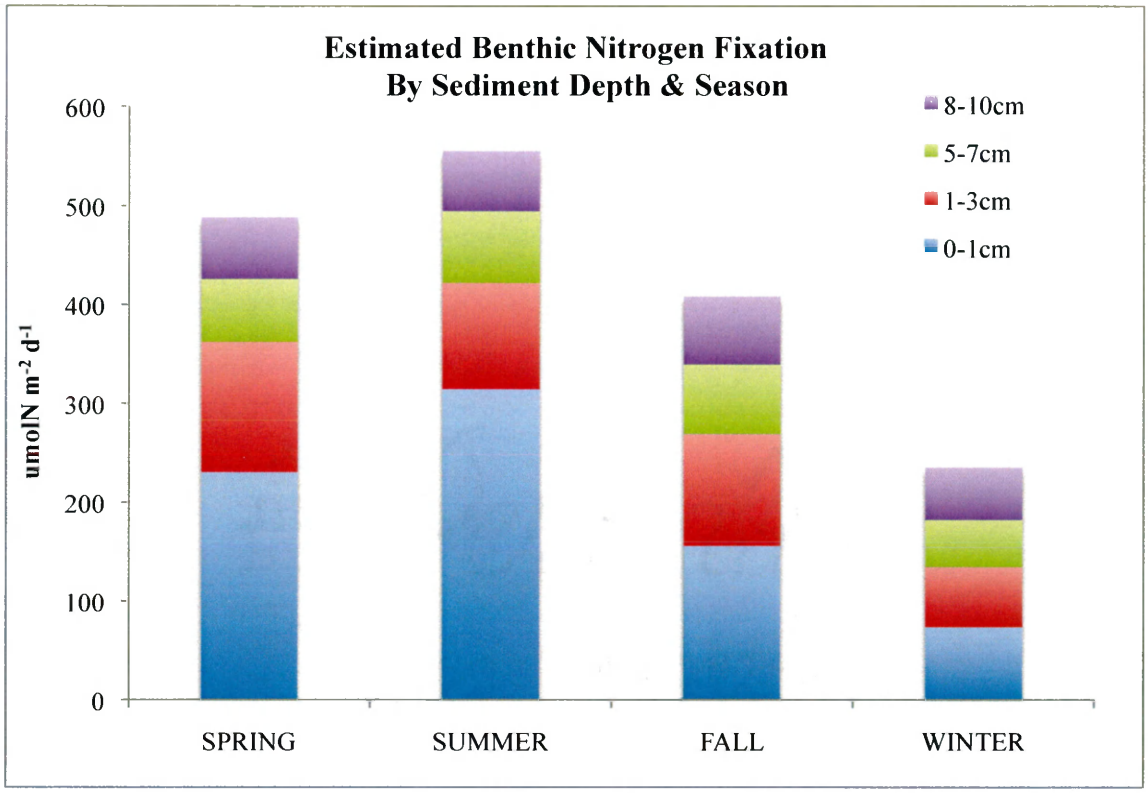


Figure 9: NFix rates per sediment section for all sites averaged per season (2009 – 2011).

Table 6: Two-way ANOVA summary table for NFix measurements at 0.5m water column depth by sediment section and season. Significant effects are in bold; asterisks are used to show interactions.

NFix rates by sediment depth						
Parameter	**Df**	**Sum sq**	**Mean Sq**	**F value**	**P Value**	
Season	3	3.51	1.17	10.79	**<.0001**	Summer>Fall and Spring>Winter
sediment depth	3	7.11	2.37	21.85	**<.0001**	0-1cm>1-3cm>5-7cm and 8-10cm
season * sediment depth	9	2.25	0.25	2.3	**0.0176**	
Residuals	191	20.71	0.108			

Estuarine wide Benthic NFix

Benthic NFix Rates were calculated for multiple water column depths in July 2010 (Figure 10A) and April 2011 (Figure 10B) with highest total rates occurring mid estuary for both seasons (July 2010, 840 umol m^2 day^{-1}, and April 2011, 960 umol m^2 day^{-1}). Figure 7 shows that NFix rates were highest in the shallow water at the upper and middle estuary sites during July 2010 and at the middle site in April 2011 but decreased with increased water depth. NFix rates per cm of sediment depth were only significantly higher in the 0-1cm section at 0.5m water depth; especially at the middle estuary site in both July and April, accounting for 69% and 58% of the total rate integrated over the 0-10 cm sediment section respectively. On average the 1-3 cm and 5-10 cm sections accounted for 25% each of the total 0-10 cm NFix rates calculated for July 2010; in April these sections accounted for 26% , and 25% of the total 0 – 10 cm rate. Results showed no significant difference between NFix rates integrated over 10 cm between spring or summer, or with changes in water column depth at any of the three sites. NFix rates integrated over 10cm showed a significant negative correlation with sediment mineralization rates (measured in July and April) (Figure 11A) and sediment NH_4^+ (measured in July), (Figure 11B). Surface sediment NFix (0-1cm) from all depths showed a significant positive correlation with benthic chlorophyll a (Figure 11C).

Denitrification rates, measured by the Piehler lab (UNC-IMS) concurrently with NFix as part of the larger DCERP study, at 0.5m and 3.0m water depths (DCERP Final Report 2012), were compared to NFix rates measured in surface (0 – 1cm) sections. Gross denitrification was calculated as DNF rates measured by membrane inlet mass

spectrometry (MIMS) plus NFix rates measured using ARA. NFix rates as a percentage of gross denitrification, shown in Figure 12, demonstrate that during the summer and spring and especially in the mid estuary NFix represented as much as 40% of gross DNF rates.

Figure 10: NFix rates per sediment section measured at multiple water column depths from three estuarine regions (up, mid, low) in July 2010 (A) and April 2011 (B) at multiple sediment depths.

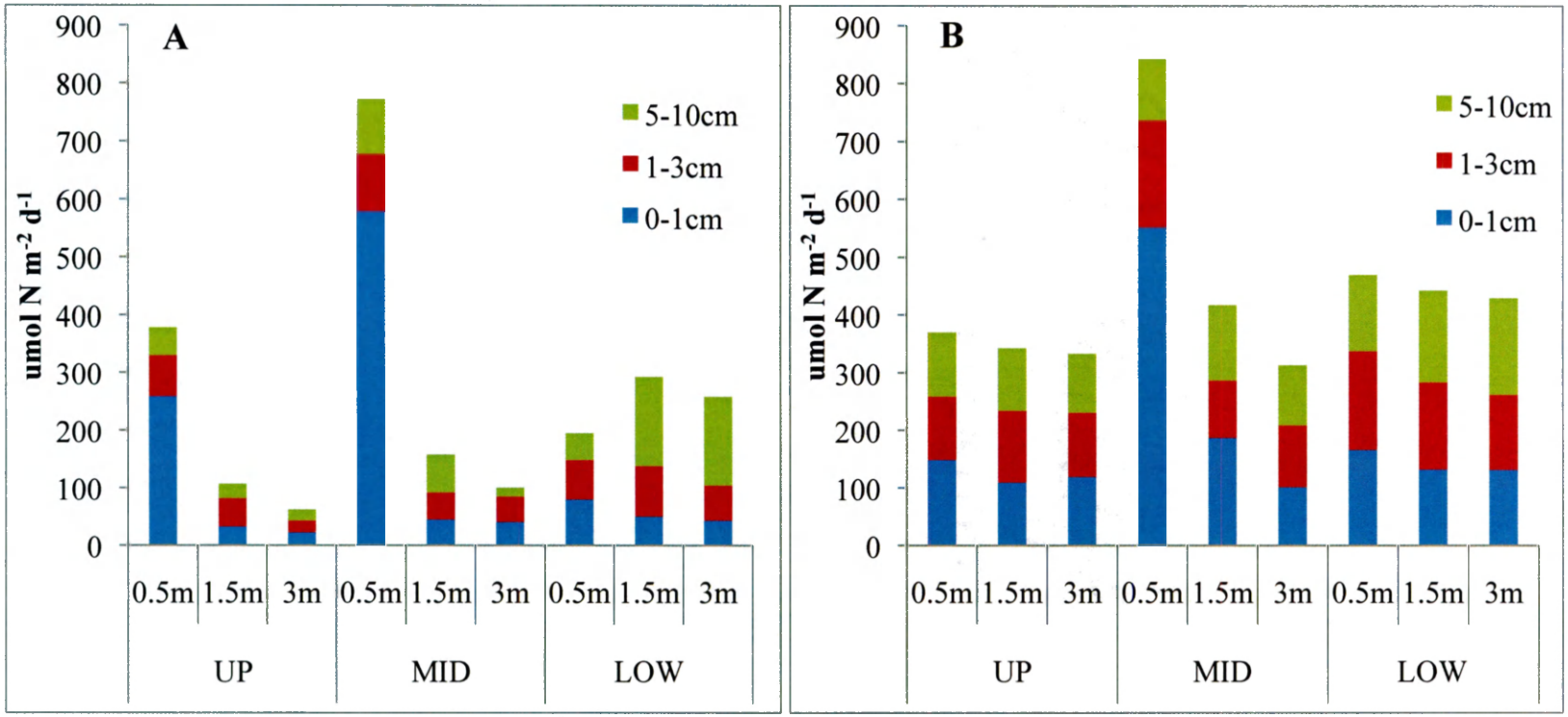
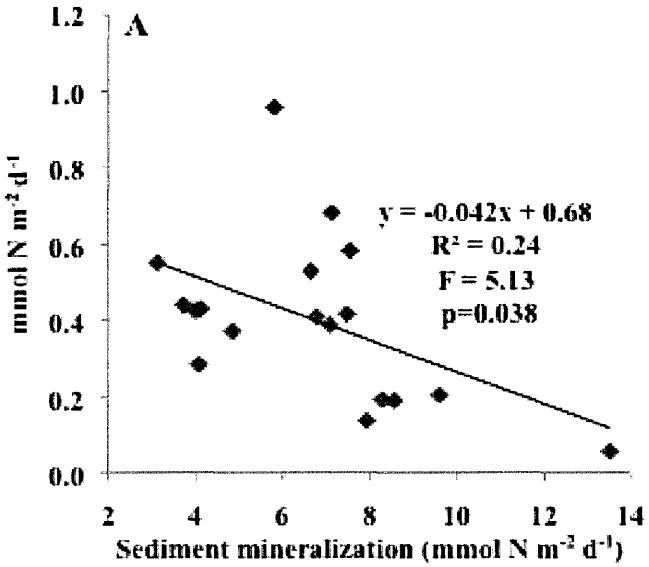
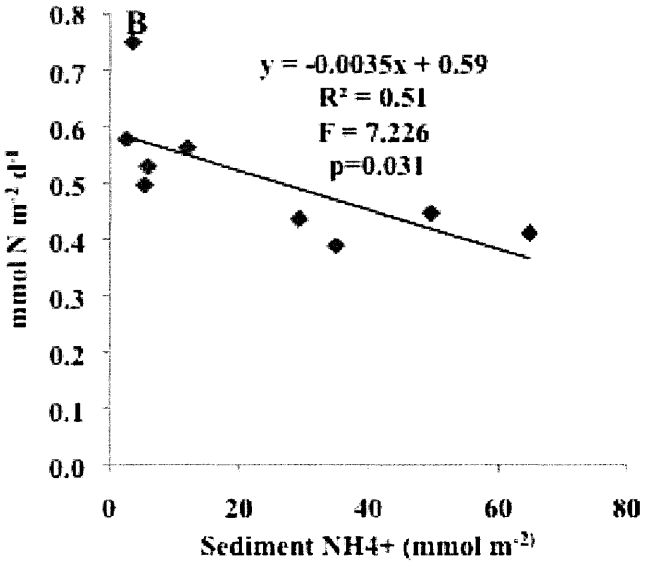
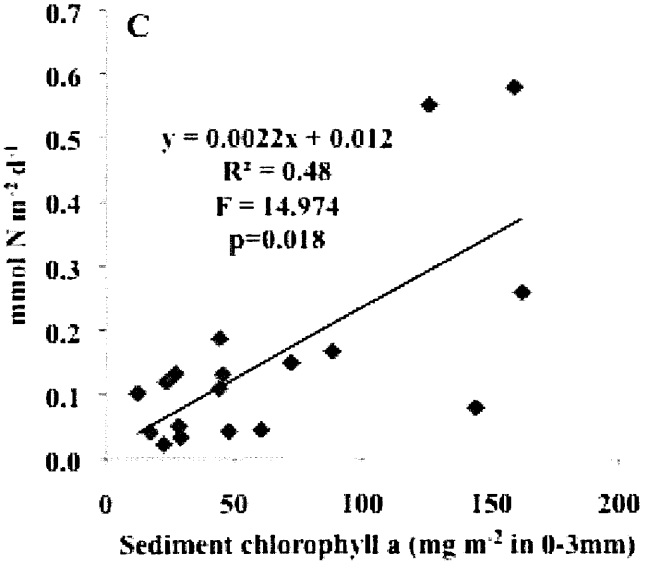


Figure 11. Sediment NFix regressed with (A) sediment mineralization rates (mmol N m^{-2} d^{-1}) (July 2010 and April 2011) and (B) sediment NH_4^+ (0-10cm) (mmol m^{-2}) (July 2010 only) and (C) sediment chlorophyll a (mg m^{-2} in 0-3mm) (July 2010 and April 2011).


A
y = -0.042x + 0.68
R² = 0.24
F = 5.13
p=0.038
mmol N m-2 d-1
Sediment mineralization (mmol N m-2 d-1)


B
y = -0.0035x + 0.59
R² = 0.51
F = 7.226
p=0.031
mmol N m-2 d-1
Sediment NH4+ (mmol m-2)


C
y = 0.0022x + 0.012
R² = 0.48
F = 14.974
p=0.018
mmol N m-2 d-1
Sediment chlorophyll a (mg m-2 in 0-3mm)

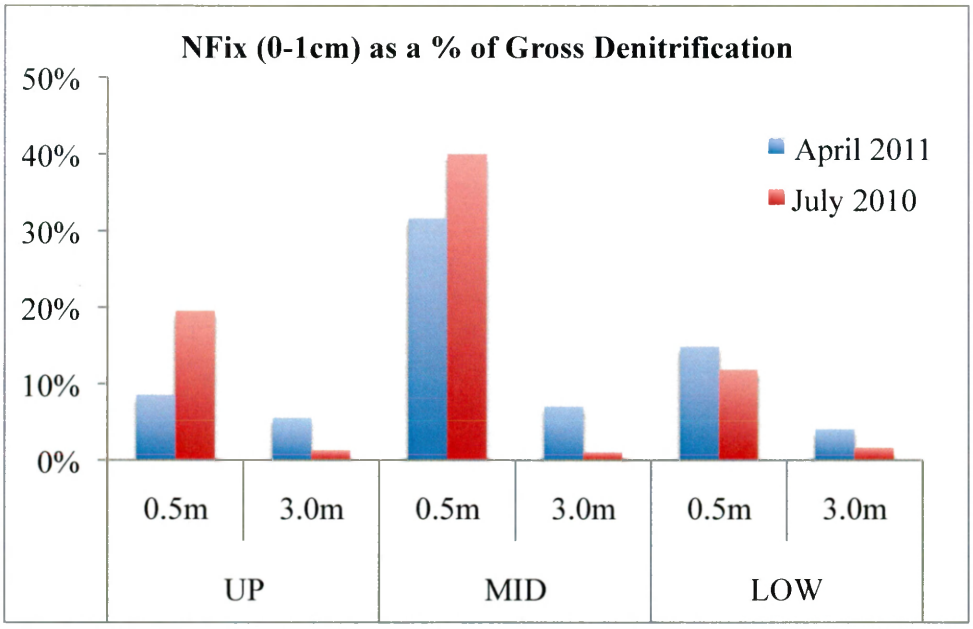


Figure 12: Benthic N-fixation (0-1cm depth horizon) as a percentage of estimated gross denitrification measured at three stations (0.5m and 3.0m water depth) in July 2010 and April 2011.

Salt Marsh NFix

NFix was investigated in salt marsh sediment to depths of 5cm and on standing shoots of marsh grass. All stations were dominated by *Spartina alterniflora* except Pollocks Point station 3 where *Distichlis spicata* was the dominant macrophyte. Epiphytic NFix, calculated to represent a square meter of stem, showed significantly higher rates than those observed in the sediment samples. Rates of epiphytic NFix ranged from 1-865 umol N m^{-2} h^{-1}; significantly ($p<0.001$) higher rates of NFix were seen at Traps Bay (Figure 13) compared to Pollocks Point (Figure 14). At Traps Bay the highest rates occurred at station 3, ranging from 114-865 umol m^{-2} hr^{-1} and significantly higher than at station 1 and 2. There was no significant difference between NFix measured during different time periods of the day at Traps Bay. All epiphytic NFix rates

at Pollocks Point were less than 5 umol m^{-2} h^{-1}, with no significant difference between stations or incubations times.

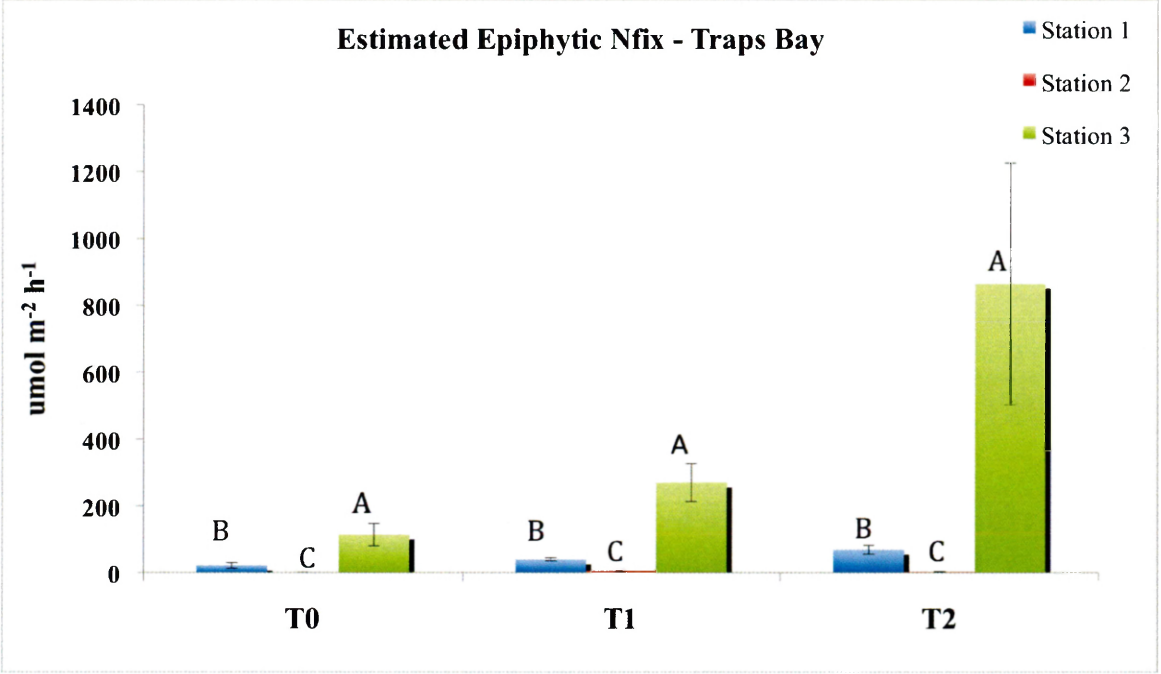


Figure 13: Estimated epiphytic NFix rates from Traps Bay from three stations at three time intervals (T0 - 7:30am-11am, T1 - 1pm-4:30 pm, and T2 - 6:30-10pm). Error bars indicate standard error. Significant differences represented by difference in letter coding.

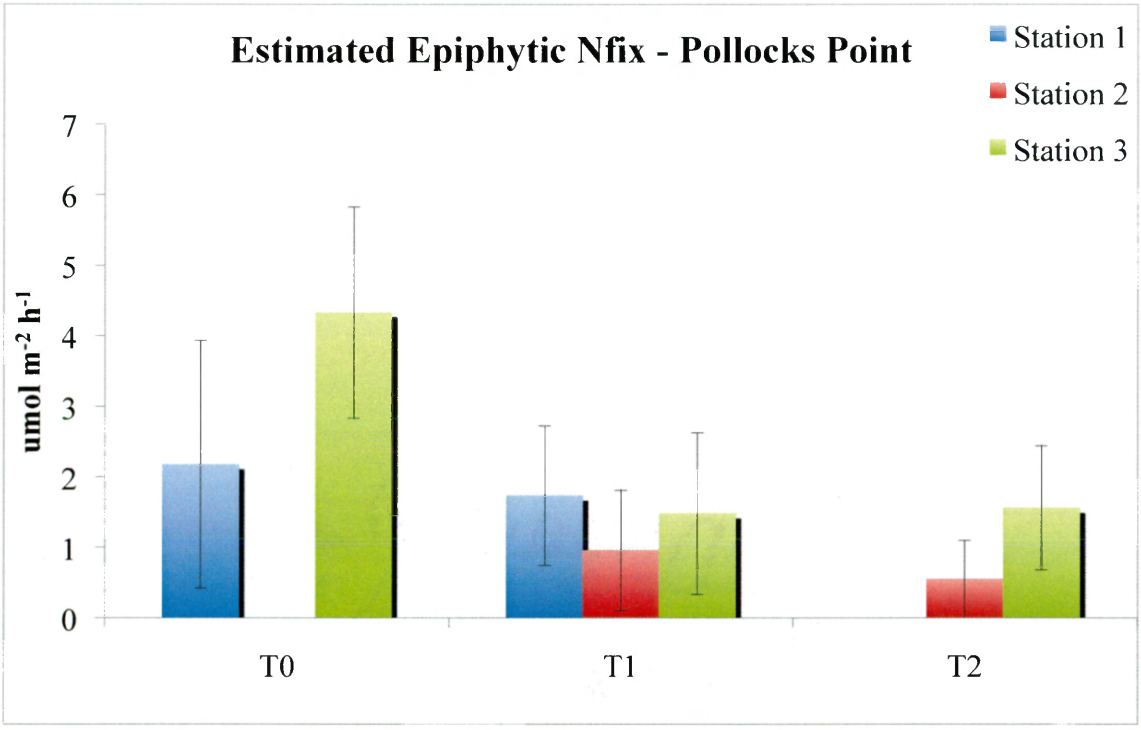


Figure 14: Estimated epiphytic NFix rates from Pollocks Point from three stations at three time intervals (T0 - 7:30am-11am, T1 - 1pm - 4:30 pm, and T2 - 6:30 - 10pm). Error bars indicate standard error.

Salt marsh sediment NFix rates (Figure 15) ranged from 0 – 140 umol m^{-2} h^{-1}. At Pollocks Point, NFix rates were significantly higher at station 1 (0.0m elevation) ($p<0.0001$) compared to station 2 (0.18m) and 3 (0.36m) with no significant differences between incubation times. NFix was detected only in the 4-5 cm section at station 2 and rates were below detection limits for all incubation times at station 3, the *Distichlis spicata* sediments with the highest elevation and least tidal inundation. Sediment NFix rates were significantly higher at Traps Bay ($p<0.0001$) than at Pollocks Point, though all of the stations at Traps Bay and two of the stations at Pollocks Point were dominated by *Spartina*. The stations at Traps Bay were all 0.22m or less in elevation, along with the

two *Spartina* stations at Pollocks Point. Station 3 at Traps Bay had significantly ($p<0.007$) higher rates of NFix than station 1 and 2 in the surface 0-1 cm section. NFix rates measured at station 1 and 2 at Traps Bay were less than 8 umol m^{-2} h^{-1}

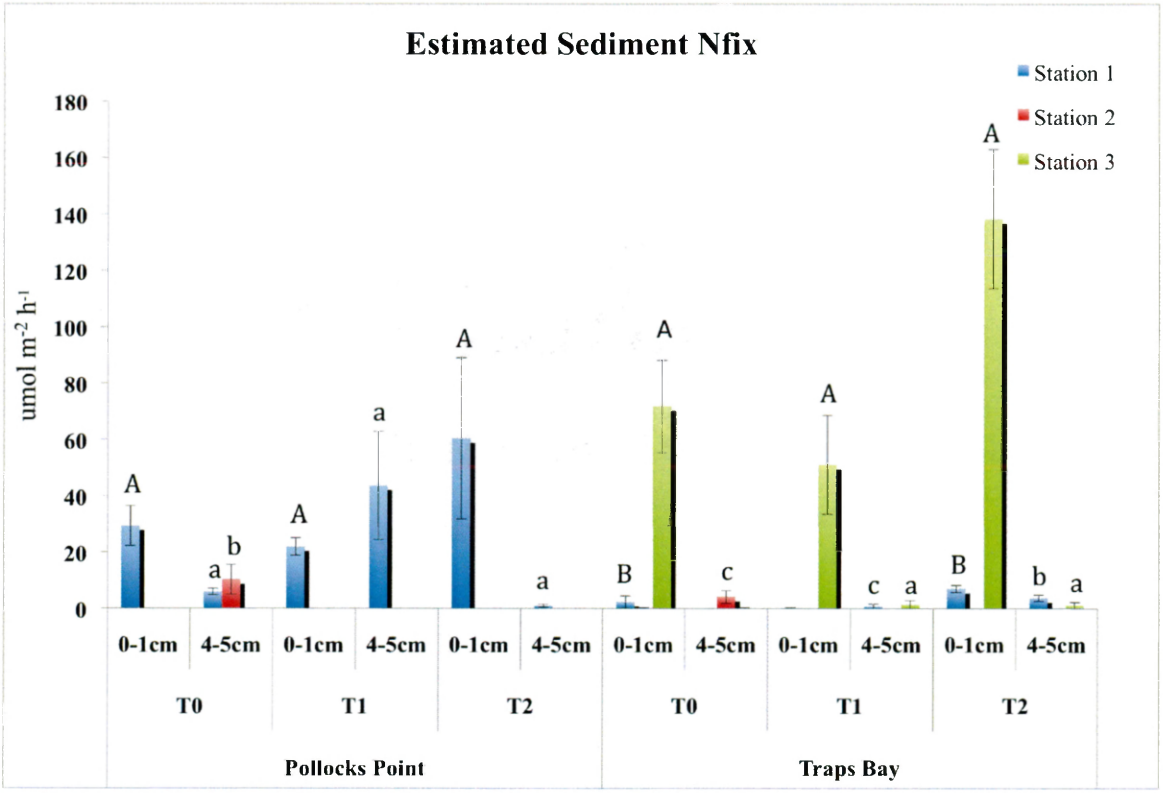


Figure 15: Estimated sediment NFix rates from Pollocks Point and Traps Bay from three stations at three time intervals (T0 - 7:30am-11am, T1 - 1pm-4:30 pm, and T2 - 6:30-10pm) and two sediment depth sections (0-1cm and 4-5cm). Error bars indicate standard error. Significant differences represented by difference in letter coding and cases.

Discussion

Spatial and temporal patterns

Based on results from this study, we supported the hypothesis that benthic NFix will increase as a function of water column temperature. Benthic NFix rates varied seasonally and annually in the NRE. NFix rates were positively correlated with temperature, peaking in the warmer months with some variation with depth, though peak rates in the lower estuary were observed in the fall. Fall water temperatures at all sites were lower than summer but on average, 2-3°C warmer than spring. NFix is known to vary seasonally and has been shown in other studies to correlate positively with temperature (Herbert 1975, Howarth et al. 1988b, Foster et al. 2009, Bertics et al. 2012).

We had hypothesized that NFix rates would be highest in areas of lowest light attenuation and lowest sediment NH_4^+. In the lower estuary sites, which have the lowest light attenuation in the NRE, NFix peaked during fall in 2009 and 2010. Results of DCERP I showed a significant relationship between % incident light and benthic chlorophyll for all sites in the NRE. We showed a positive relationship of benthic NFix rates and benthic chl a suggesting that NFix rates will also vary with light.

In this study NFix rates in both shallow and deep water column sites were negatively correlated with sediment ammonium (July 2010) and also with sediment mineralization, which produces ammonium, at multiple water column depths (July 2010 and April 2011). Numerous studies have similarly looked at the effects of pore water and

extractable ammonium on NFix with some results suggesting it inhibits NFix (Capone and Carpenter 1982, Capone 1988) and others showing no effect (Capone 1988, McGlathery et al. 1998).

In this study NFix rates in the NRE exhibited a negative correlation with organic matter content during summer. In sediments with high rates of mineralization and decomposition of organic matter, one would expect high rates of sulfate reduction. Marino et al (2003) showed that high sulfate concentrations, often found in estuarine sediments, can slow the growth rate of NFixing cyanobacteria, making the sediments a more favorable environment for other NFixing organisms that use sulfate as an energy source. In deep anoxic sediments (water depth ~28m) with high rates of observed sulfate reduction, NFix rates as high as 220 umol m^{-1} d^{-1} integrated over 25cm sediment depth were measured by Bertics et al. (2012). The presence of organic matter can promote oxygen consumption, thereby alleviating the problem of oxygen inactivating the nitrogenase enzyme (O'Neil and Capone 1989). Results of this study highlight opposing drivers in the benthos; temperature increases benthic NFix rates as well as organic matter decomposition and mineralization, but mineralization and organic matter are shown here to be negatively related to NFix rates.

The depth profiles of benthic NFix in this present study show that NFix occurred in shallow (0.5 m MSL) and deeper water (3 m) at sediment depths up to 10 cm below the surface. Bertics et al. (2012) measured highest rates of NFix in the first 10cm, with rates decreasing with sediment depth in each season. In recent studies, high rates of NFix have been identified in deep water estuarine sediments (Fulweiler et al. 2007), bare and eelgrass dominated shallow water coastal sediments (McGlathery 1998, Cole and

McGlathery 2012), and shallow water sediments with and without microbial mat populations (Bertics et al. 2010, Steppe and Paerl 2005). A comparison of rates observed in this study with those of other studies are shown in Table 7. The high organic matter content and low oxygen levels of some deeper sediments may provide a favorable environment for heterotrophic NFixers including sulfate-reducing bacteria over phototrophic NFixers (Fulweiler et al. 2008, Bertics et al. 2012).

NFix has been shown to offset losses of fixed N from sediment due to denitrification and anammox (Bertics et al. 2012) and in some estuaries the benthos has been shown to switch from a net N_2 source when DNF rates are higher than NFix, to a N_2 sink when organic matter inputs decrease and stimulate NFix relative to DNF (Rao and Charette 2012, Fulweiler et al. 2007). As in the Fulweiler et al study (2007) results of this study similarly show NFix rates decreasing as organic matter increased. In the NRE NFix and DNF varied seasonally, though DNF was higher in deeper water sediment and NFix lower. NFix may offset as much as 40% of fixed N loss through denitrification in shallow sediments in the NRE.

Table 7: Integrated NFix rates in NRE sediments compared to other studies.

Location	Depth of integration (cm)	NFix rates umol N m^{-2} d^{-1} (using 4:1 ratio C_2H_2:N_2, which corresponds to 2:1 C_2H_4:NH_3)
Eelgrass rhizosphere (McGlathery et al. 1998)	0 to 14	71 to 430
Eelgrass meadow sediments (Cole and McGlathery 2012)	0 to 5	146 to 390
Coastal bioturbated sediments (Bertics et al 2010)	1 to 10	150 to 2430
Coastal bioturbated sediments – includes microbial mat (Bertics et al 2010)	0 to 10	160 to 8050
Coastal sediments to 25cm depth (~28m water depth) (Bertics et al. 2012)	0 to 25	80 to 220
Exposed lagoon sediments (Charpy-Roubaud et al. 2001)	0 to 2	10 to 570
Subtidal sediments (Fulweiler et al. 2007)	0 to 30.5	600 - 15,600
Intertidal microbial mat (Steppe & Paerl 2005)	0 to 5	1630 ± 1150
New River Estuary sediments (this study)	0 to 10	92 to 1860

We hypothesized that benthic NFix would display strong seasonal variation and be an important source of new N to the NRE especially during summer months. On an estuarine-wide basis NFix contributed approximately 19% in spring, 16% in summer, and 13% annually to total N loads including those from the MCBCL waste water treatment facility, wet and dry atmospheric deposition, exchange with the coastal ocean at Onslow Bay and watershed inputs (Table 8). Total N loads to the NRE were calculated as part of the larger DCERP study to assess the importance of watershed processes to estuarine nutrient loading (Anderson et al. 2012). The largest allochthonous input of N to the NRE is from agricultural and off-base watershed inputs coming down the New River. Comparatively, benthic NFix in Hog Island Bay, a shallow lagoon on the oceanside of the

Eastern Shore in Virginia, contributed 10% compared to allochthonous sources of N (Anderson et al. 2010). This study shows that benthic NFix is an important source of new biologically available N to the NRE, helping to support benthic primary production and offset N losses through denitrification.

Table 8: Allochthonous sources of N to the NRE and NFix inputs annually and seasonally using averages for NFix (2009-2011) and other sources (1998-2011) (DCERP Final Report 2012).

Sources	Annual Input (TN kg/year) (%)	Spring Input (TN kg/season)	Summer Input (TN kg/season)
Off-Base	505,515 (55%)	94,983 (47%)	129,322 (55%)
MCBCL	63,644 (7%)	15,911 (8%)	15,911 (7%)
WWTF	54,942 (6%)	13,857 (7%)	12,989 (6%)
Onslow Bay	120,451 (13%)	28,899 (14%)	26,496 (11%)
Direct Atm Dep	49,628 (5%)	12,407 (6%)	12,407 (5%)
NFix	**122,291 (13%)**	**37,802 (19%)**	**38,027 (16%)**
Total	916,473,670	203,860,598	235,153,954

Salt marsh NFix in the NRE

Salt marsh NFix rates were extremely variable. The majority of NFix in the two salt marshes studied in the NRE was attributed to epiphytes on the macrophyte shoots. Epiphytic NFix at Traps Bay was highest at night, although not significant, and was similar to measurements made with standing dead stems treated with seawater in both natural and transplanted *S. alterniflora* salt marshes in North Carolina (Table 9; Currin and 1998, Moisander et al. 2005). Low rates of NFix at Pollocks Point are most likely

due to the relatively higher elevation at station 2 (0.18m) & 3 (0.36), as similar rates were observed at Traps Bay station 2 (0.22m). It is unclear why shoreline rates of epiphytic NFix at station 1 (0.0m) were so low.

Table 9: Measured rates of epiphytic NFix in North Carolina *S. alterniflora* salt marshes.

Location	umol C_2H_4 m^{-2} h^{-1}	umol N m^{-2} h^{-1} (4:1 ratio C_2H_2:N_2)
Pollocks Point	0 – 8.6	0 – 4.3
Traps Bay	0 - 1730	0 – 865
Natural Salt Marsh, NC (Currin & Paerl 1998)	5 – 140	2.5 – 70
Transplanted Salt Marsh (Currin & Paerl 1998)	10 - 330	5 - 165
Natural Salt Marsh, NC (Moisander et al. 2005)	0.7 - 2060	0.35 - 1030

Sediment NFix rates measured in the salt marsh sediments were significantly lower than epiphytic rates (p=0.0005). The rates measured in the *Spartina alterniflora* dominated marsh were higher in the evening although not significant, during the simulated high-tide, and lowest during the daytime incubation in the surface (0-1cm) section, due possibly to oxygen inhibition during active photosynthesis. Similar patterns of rates were found to occur in *Salicornia virginica* sediments taken in a salt marsh in Mission Bay (CA), low during the day and highest at night (Mosemon 2008). Surface sediments in a *Spartina alterniflora* marsh in North Carolina showed both daytime and nighttime peaks in NFix with nighttime peaks in sediments dominated by non-heterocystous cyanobacteria and daytime peaks in sediments dominated by heterocystous and coccoid cyanobacteria

(Currin et al. 1996). Surface sediments at station 3 (Traps Bay) exhibited the highest rates of NFix, which may be attributed to lower stem density and above ground biomass, which would block light from reaching the surface.

Although much is known about interactions between nitrogen fixers and *Spartina alterniflora* (Whiting *et al.* 1986, Currin et al. 1996, Currin & Paerl 1998, Tyler et al. 2003), less is known about patterns of NFix with other vascular plant species including *Juncus roemerianus,* which along with *S. alterniflora.,* dominate NRE marshes. Although approximately 21% of the shoreline of the NRE was identified as marsh, due to the small marsh water shoreline interface, constrained drainage, and limited delivery of groundwater N, it is not considered an important source of N to the NRE (DCERP Executive Summary 2009). Hourly NFix rates measured in this experiment were highly variable between sites and times of day but were on average comparable to benthic NFix, leading us to conclude that the salt marsh may at times be an important source of N to the estuary. Other factors affecting potential export of N from the marsh include the fringing marsh ecotype, which has a small marsh water shoreline interface, the constrained drainage due to dampened tidal amplitude and limited delivery of groundwater N (DCERP Annual Report 2011).

Conclusion

Benthic NFix was highest in surface sediments in shallow water and in warmer months during our study. We sampled seasonally along the estuarine gradient at multiple water depths (with a range of light availabilities) and multiple sediment depths. Rates of NFix were shown to vary positively in response to light levels and temperature. Although highest rates of N-fixation were in the top 0 – 1 cm, a substantial portion occurred down to 10 cm in shallow and deep water samples. There was a negative correlation of NFix rates with both sediment NH_4^+ concentration and organic matter content, though this negative relationship may only be related to phototrophic NFixers. N-fixation rates varied seasonally and were a significant source of autochthonous N to the NRE, contributing up to 19% of total inputs during spring and supplying up to 40% of N removed by denitrification.

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CHAPTER 2

Potential benthic nitrogen fixers in the New River Estuary, NC: Identification and Distribution

Introduction

In coastal marine systems, nitrogen (N) availability is often the key factor controlling primary production (Dugdale and Goering 1967; Ryther and Dunstan 1971; Nixon 1992; Nixon et al 1996; ,Valiela, 1983; Taylor et al. 1995). Nitrogen may be supplied to coastal estuaries from sources outside (allochthonous) or within (autochthonous) the estuary. Allochthonous sources include agricultural, forest, and urban runoff, groundwater discharge, sewage and industrial waste and atmospheric deposition (Andersen et al. 2007, Howarth et al. 2011). Autochthonous sources include nitrogen fixation (NFix) (Paerl and Zehr 2000; Gardner 2006), the only source of new autochthonous N, dissimilatory nitrate reduction to ammonium (DNRA) (An and Gardner 2002; Gardner 2006), and remineralization of dissolved and particulate organic N (Cowan and Boynton 1996).

Nitrogen fixation (NFix), the reduction of atmospheric N_2 to the biologically available form NH_4^+, can be an important source of new N to marine systems and can help to offset losses via denitrification (Howarth et al., 1988; Capone 1988, 2001; Galloway et al., 2004). Pelagic, benthic and epiphytic cyanobacteria along with numerous other autotrophic and heterotrophic bacterial and archael species have been found to fix N across a broad range of salinities in estuarine ecosystems (Capone 1988; Paerl, 1996; Bertics et al., 2010; McGlathery et al. 1998; Carpenter and Capone 2008; Cole and McGlathery, 2012; Severin et al., 2012). Planktonic N fixation is most often

associated with blooms of cyanobacteria that are generally found in tropical and subtropical oceans and are rarely observed in estuaries even though most temperate estuaries are N limited (Howarth et al. 1988, 1999; Paerl & Zehr 2000; Zehr et al., 2001, Carpenter and Capone 2008). However, recent studies have shown relatively high rates of NFix in both vegetated (McGlathery, 2008) and bare estuarine sediments (Fulweiler, 2007; Bertics et al., 2010). NFix can be a major source of N to benthic primary producers, including seagrasses, macroalgae, and benthic microalgae (BMA), important autotrophs in shallow coastal ecosystems (Cahoon, 1999; Nixon, 2001, Cole and McGlathery 2012; Carpenter and Capone 2008).

NFix is carried out by a variety of heterotrophic and autotrophic microorganisms possessing the nitrogenase enzyme complex. Organisms capable of fixing nitrogen (diazotrophs) include Archaea and Bacteria, ranging from unicellular (*Crocosphaera*), non-heterocystous filamentous (*Trichodesmium*), and heterocystous cyanobacteria (*Richaelia* spp.) to Proteobacteria (*Desulfovibrio* spp.) and Firmicutes (*Paenibacillus* spp.) (Young, 1992; Zehr et al., 2003; Raymond et al. 2004, Moisander et al., 2007; Latysheva et al., 2012). The nitrogenase enzyme complex, which is responsible for breaking the N-N triple bond and is permanently inactivated by the presence of oxygen (Zehr and McReynolds 1989), is made up of two polypeptide subunits; the molybdenum-iron (MoFe) protein which is encoded by the *nifD* and *nifK* genes, and the dinitrogen reductase (Fe) protein encoded by the *nifH* gene (Zehr and McReynolds 1989, Rees and Howard 2000). The availability of these metals in an ecosystem may regulate rates of NFix (Vitousek 2002). The most evolutionarily conserved, and extensively characterized of the genes is *nifH*, which encodes the Fe-only protein subunit of the enzyme (Postgate,

1982; Young, 1992; Latysheva et al., 2012). The use of degenerate polymerase chain reaction (PCR) primers to amplify the *nifH* gene, as first described by Zehr and McReynolds (1989), along with quantitative reverse transcriptase PCR (RT-qPCR) are some of the tools currently being used to study *nifH* gene diversity and expression patterns in aquatic microbial communities. PCR is used to identify the organisms possessing the gene and RT-qPCR to quantify the number of *nifH* gene transcripts that are actively being expressed (Short and Zehr, 2005). In the Chesapeake Bay where benthic NFix is not thought to be an important source of N due to low rates or absence of detection, diverse assemblages of *nifH* genes have been identified. *NifH* sequences obtained using PCR amplification from highly reduced sediments of anoxic mud taken in the mid-portion of the Chesapeake Bay clustered closely with each other and with known anaerobic organisms (Burns, 2002), but only two phylotypes were detected expressing the gene, both related to cyanobacterial *nifH* genes (Jenkins et al., 2004; Short and Zehr, 2005). Sulfate reducing bacteria (SRB) have been identified as important contributors to NFix in bioturbated intertidal lagoon sediments (Bertics et al. 2010) and also in intertidal microbial mats (Steppe and Paerl, 2002) using RT-PCR or PCR along with the use of metabolic inhibitors, specifically sodium molybdate, to infer the contribution of SRB's to NFix rates. SRB's, which are responsible for much of the remineralization of organic matter in anoxic sediments, have also been found to be major contributors to NFix in sea grass rhizospheres (Capone, 1988; Welsh et al., 1996b).

Nitrogenase enzyme activity and, therefore, the rate of NFix is influenced by the energy demands of NFix (16 moles of ATP per mole of N_2 fixed) and by a variety of environmental factors, including light, oxygen, temperature, salinity and ambient

ammonium concentrations (Howarth et al., 1988; McGlathery, 2008; Kranz, 2000). It has been shown that intertidal microbial mats, structurally dominated by cyanobacterial NFixers, exhibit a shift in active diazotrophic community structure and nitrogenase activity with changing salinities. Whereas cyanobacterial *nifH* transcripts comprised the majority of clone libraries at ambient salinities (33 PSU) gammaproteobacterial and deltaproteobacterial *nifH* transcripts dominated at higher salinities (66 and 165 PSU) (Severin and Stal, 2010; Severin et al., 2012). Because the nitrogenase enzyme is permanently inhibited by the presence of O_2, phototrophs have developed numerous strategies to protect the enzyme from oxygen produced during photosynthesis, including segregation of active nitrogenase into specialized cells called diazocytes (El-Shehawy et al., 2003), development of heterocysts, specialized cells for NFix that contain a protective wall and have only photosystem I (Schmetterer, 1994; Berman-Frank et al., 2001), and by creation of a temporal window for NFix by down regulating oxygenic photosynthesis during mid-day (Staal et al., 2003). The presence of ammonium has been presumed to inhibit NFix because less energy is required to assimilate ammonium than is required to synthesize nitrogenase and fix nitrogen (Howarth, 1988). Capone and Carpenter (1982) showed that NFix rates could be stimulated in salt marsh sediments by the removal of interstitial ammonium (Carpenter and Capone 1982, Capone 1988); however, pore water ammonium with *in situ* concentrations of up to 650 uM had no negative effect on NFix in sediment cores containing live eelgrass collected from the Limfjord, Denmark (McGlathery et al., 1998).

Previous studies on estuarine NFix have tended to focus on cyanobacterial dominated microbial mats or sediments populated by seagrasses and marsh plants

(Carpenter and Capone, 2008). The goal of this present study was to characterize the spatial and temporal variation of benthic NFix across an estuarine salinity gradient and to determine the variation in benthic diazotrophs assemblage with location and water and sediment depth. Both nitrogenase activity measurements and molecular characterization (PCR of *nifH* fragments) were used to determine the relative contributions of autotrophic and heterotrophic N-fixers to the benthos.

Methods

Site Description

The New River Estuary (NRE) (Figure 1) is located in southeastern North Carolina, and is mostly surrounded by Marine Corps Base Camp Lejeune (MCBCL) with the city of Jacksonville located at the head of the estuary. The NRE is characterized as a shallow water body with over half the estuary having a depth of less than 2m at mean sea level (MSL; NAVD88). It has an estuarine surface area of approximately 88 km^2 and is fed by a watershed estimated to be 462 square miles (Tomas et al., 2007). The average flushing time of the NRE is 64 d as the mouth of the estuary is a narrow inlet constrained by barrier islands, but changes in climactic conditions lead to variability in the flushing time (Ensign 2004). The tidal range, which on short timescales averages approximately 30 cm, ranges from approximately 43cm near the mouth of the estuary to 13cm towards the head (per Carolyn Currin comm.)

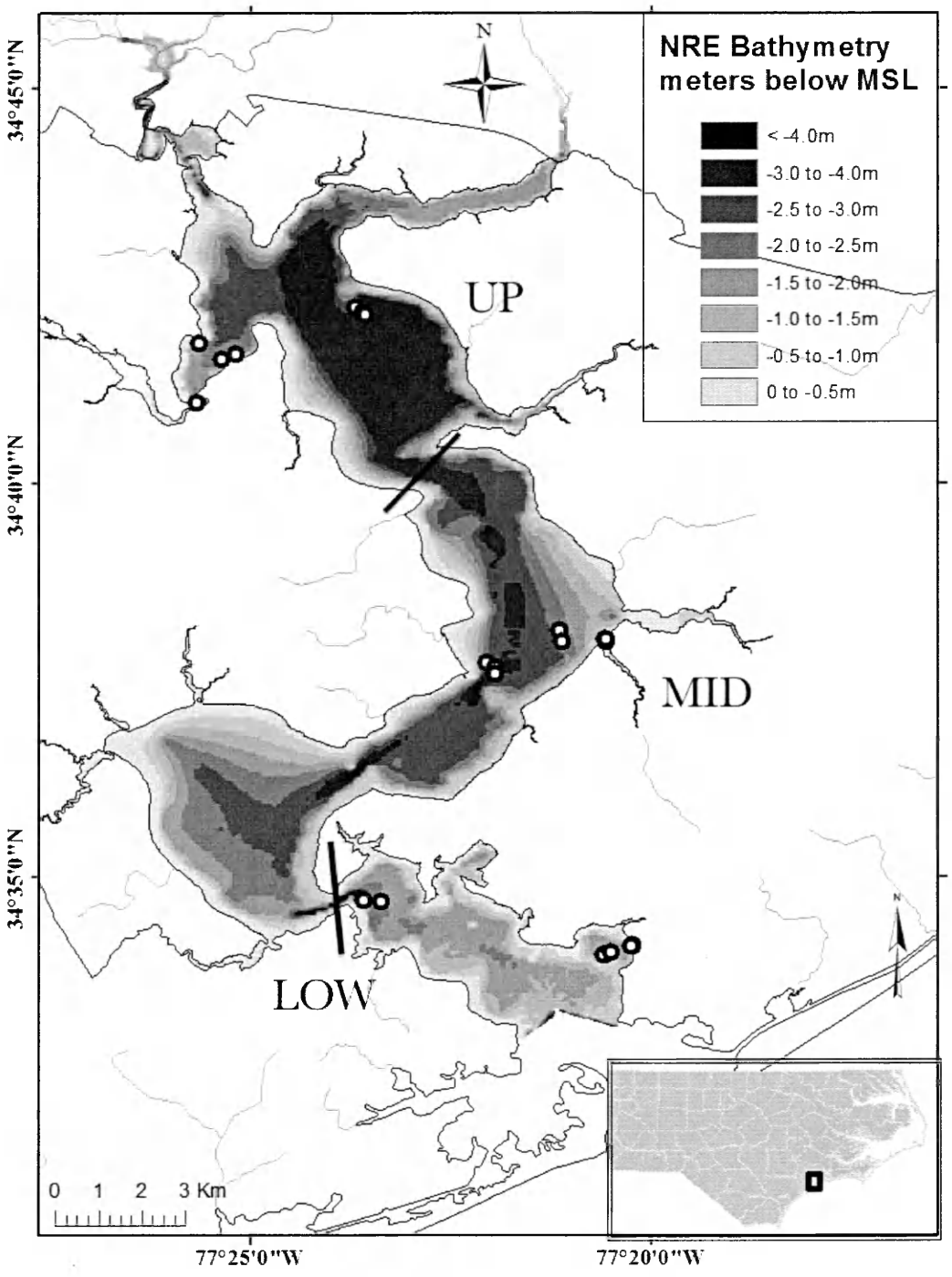


Figure 1: NRE bathymetry (MSL; NAV88) and depth experiment sampling stations in the upper, middle, and lower regions of the estuary (McNinch 2009).

The NRE exhibits moderate eutrophication and strong gradients of dissolved inorganic nitrogen (DIN), light attenuation, benthic and pelagic chlorophyll *a* (chl *a*), and chromophoric dissolved organic matter (CDOM). The down-estuary gradient for benthic and water column chl *a*, extractable NH_4^+, and dissolved organic and inorganic nitrogen (DON, DIN) with highest values up-estuary suggests that on an annual basis the New River watershed is the main external source of nitrogen to the estuary (Anderson et al, 2012). Major allochthonous sources of N to the watersheds that drain to the NRE include agricultural runoff, confined animal feeding operations (CAFOs), forest clear-cutting, urban land use, atmospheric deposition, and sewage spills (Anderson et al., 2012).

Hydrologic forcing, especially fresh water discharge, has been shown to play an important role in the NRE, delivering N, which has been shown to limit primary production, and chromophoric dissolved organic matter (CDOM), which limits light availability (Anderson et al., 2012). When comparing a slightly below average rainfall year (2009) and an average year (2010), algal bloom density and frequency were highest in 2009 when high N loads were combined with moderate flushing as opposed to 2010 when increased flushing lowered the residence time of the N loads. Autochthonous N regenerated internally within the estuary was more important during drier summer periods when allochthonous N sources were at a minimum to support phytoplankton blooms (Anderson et al., 2012).

A highly productive benthos, as indicated by high sediment chlorophyll *a*, suggests that benthic processes are likely to play an important role in retaining and transforming nitrogen and reducing the impacts of nitrogen enrichment to the system. Studies from Anderson et al. (unpub. data) have demonstrated that during summer, when

allochthonous inputs are usually at a minimum, N produced by benthic ammonification, including NFix, can potentially supply more N to the water column than the New River and Southwest Creek watersheds, the primary allochthonous sources of nitrogen to the NRE; however where sufficient light is available benthic autotrophs are responsible for sequestering much of the ammonified N.

Sampling and site characterization

For determination of spatial variation of benthic NFix between three regions and three water depths of the NRE, sediment cores were collected in July 2010 at triplicate sites in the upper, mid, and lower estuary randomly selected from GIS maps of 0.5 m 1.5 m, and 3.0 m MSL isobaths based on bathymetric surveys conducted in 2009, (see Chapter 1 for details).

Sediment samples were analyzed for sediment organic content and bulk density, extractable nutrient concentrations (NO_x and NH_4^+), and benthic chl *a* (Table 1). Bulk density was determined in samples dried at 50°C to constant weight (±0.01 g), followed by combustion at 500°C for 5 hours to determine percent organic matter. Extractable nutrient concentrations were determined in sediment samples by shaking for 1-hour with 2M KCl (2 volumes KCl : 1 volume sediment), centrifuged, and filtered (0.45 um Supor Acrodisk syringe filter) prior to analysis for NH_4^+ and NOx on a Lachat nutrient autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). Samples for analysis of benthic chl *a* were taken with a 5 ml syringe to a depth of 3 mm, placed in 20 ml centrifuge tubes on site, kept on ice in the dark and frozen until analyzed. Samples were then extracted with 10 ml of 90% acetone in deionized water, vortexed for 30 seconds,

sonicated for 30 seconds and placed into the freezer for 24 hours. The extractant was filtered through a 0.45μm PTFE Acrodisc filter and read on a spectrophotometer (Beckman Coulter DU 800 Spectrophotometer) at 665, and 750 nm for determination of chlorophyll-*a*.

Table 1. Sediment and Site characterization experimental methods.

Analyses	Method/Instrument	Reference
Sediment Organic Content	Loss on ignition (500°C)	
Bulk density	Dry @ 40°C	
Sediment Nutrients	Potassium chloride-extraction	Kenney and Nelson 1982
Benthic Chl *a*	Acetone Extraction/ spectrophotometry; Shimadzu UV-1601 Spectrophotometer	Neubauer et al., 2000; Lorenzen, 1967

NFix measurements

NFix rates were measured using the acetylene reduction technique. To convert from moles of acetylene reduced to moles of nitrogen fixed, a ratio of 4:1 of moles of acetylene reduced to ethylene to moles of N_2 reduced to ammonia was used. Although this ratio has been shown to vary (Postgate 1988, Rees and Howard 2000), results comparing $^{15}N_2$ uptake to acetylene reduction in *Trichodesmium* cultures by Mulholland et al (2004) suggest that the 4:1 conversion factor provides the best approximation of gross NFix. Acetylene generated from a mixture of CaC_2 and deionized water was added to sediment subsamples in 60 ml serum bottles to occupy approximately 20% of the headspace. Blanks containing only acetylene and controls containing sediment samples

without acetylene were incubated along with the rest of the samples. No production of ethylene was observed in the control treatments suggesting that the production of acetylene was due solely to NFix.

Samples were incubated for 6 hours at in situ temperature with a light intensity of 500 $\mu E\ m^{-2}s^{-1}$ determined to be the saturating irradiance from photosynthesis vs. irradiance curves performed on similar sediment samples from the NRE (Brush 2012), and dark (see details below). After incubation the headspaces of the incubation bottles were mixed and sub-sampled. Gas samples were transferred to Hungate tubes and stored upside down in tap water and transported to VIMS for gas analysis. Ethylene concentrations were analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (220°C, oven temp 80°C). Ethylene and acetylene were separated using a 6 ft Poropack N Column with a carrier gas (ultra-pure carrier grade He) flow rate of 20 ml min^{-1}. Ethylene concentrations were determined by comparison of peak values to a three point linear regression of ethylene standards (10 ppm, 5 ppm, and 1 ppm).

Variation of NFix rates in the light and dark

Surface sediments were subsampled (0–1 cm) twice from each core for incubation in both the light (500 $\mu E\ m^2\ s^{-1}$) and dark. Sediment samples for dark incubation were sparged with N_2 gas to produce anaerobic conditions. Both light and dark incubations were performed in an environmental chamber at in situ temperatures.

Molybdate inhibition experiment

Molybdate inhibition, as first described by Oremland and Capone (1988), involves the use of sodium molybdate to inhibit sulfate reduction in order to determine the extent to which sulfate-reducing bacteria are responsible for observed patterns of NFix (McGlathery 1998). Molybdate, a specific inhibitor of sulfate-reducing bacteria, uncouples energy production from NFix and rapidly depletes ATP pools (Oremland and Capone 1988, Taylor and Oremland 1979). In this method, molybdate is added as Na_2MoO_4 to sediment samples and acetylene reduction is measured concurrently in amended and un-amended samples (Bertics 2010, McGlathery et al. 1998).

Six sediment cores per site were collected at 0.5m water depth (MSL) in the upper, middle, and lower regions of the NRE in August 2011. Sediment cores were sub-sampled at 0-1 cm, 1-3 cm, and 5-10 cm depths. Inhibited subsamples were slurried with site water amended with 20 mM sodium molybdate (Na_2MoO_4). Surface sediments were incubated aerobically in the light at an intensity of 500 μE m^{-2} s^{-1} and dark. Deeper sediment sections were incubated anaerobically only in the dark at in situ temperatures. Uninhibited control subsamples were amended with site water only and NFix rates determined by acetylene reduction, as described above, of inhibited and non-inhibited samples.

PCR analysis

PCR amplification was used to determine general prokaryotic community composition (16S) and the microbial community with the potential to fix nitrogen (*nifH)* in sediments collected from the NRE. Sediment samples were collected from upper,

middle and lower estuarine sites at water column depths of 0.5m and 3.0m in the summer. Total microbial DNA was extracted from sediment sub-samples (0-1 cm, 1-3 cm) using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA USA) and stored at -80°C.

16S PCR

To determine general prokaryotic community composition, PCR was performed using universal 16S primers described by Turner (1999); 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 519R (5' GWA TTA CCG CGG CKG CTG 3'). Amplification reactions were carried out in a DNA Engine thermocycler (MJ Re- search Inc., Waltham, MA, USA) with reaction volumes of 25 ul and containing: 1mg/ml bovine serum albumin (BSA) (Idaho Technology, Inc., Salt Lake City, UT), 1X PCR buffer, 10 mM (each) deoxynucleoside triphosphates (dNTPs), 100 uM of both forward and reverse primers, 5 U/ul of Taq polymerase (Invitrogen), and 1 µl DNA template. Thermocycling conditions were as follows: an initial denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, chain extension at 65 °C for 2 min, with a final extension at 65 °C for 5 min. Amplified products (5 ul aliquots) were visualized by agarose gel electrophoresis (1.5% w/v), stained with ethidium bromide, viewed under a UV light source and selected for sequence analysis (approximately 510bp).

NifH PCR analysis

To analyze the *nifH* genotypes present, DNA sequences were amplified using degenerate

general primers of highly conserved regions of the *nifH* gene. A nested PCR with the internal primer pair of nifH1 (5' TGY GAY CCN AAR GCN GA 3') and nifH2 (5'ADN GCC ATC ATY TCN C 3') (Zehr & McReynolds, 1989) and the external primer pair nifH3 (5' ATR TTR TTN GCN GCR TA 3') and nifH4 (5' TTY TAY GGN AAR GGN GG 3') (Zani et al., 2000) was performed.

Amplification reactions were carried out in a DNA Engine thermocycler (MJ Research Inc., Waltham, MA, USA) with reaction volumes of 25 ul and containing: 1mg/ml bovine serum albumin (BSA) (Idaho Technology, Inc., Salt Lake City, UT), 1X PCR buffer, 10 mM (each) deoxynucleoside triphosphates (dNTPs), 100 uM of both forward and reverse primers, 5 U/ul of Taq polymerase (Invitrogen), and 2 µl DNA template for the external primer reaction and 1uL DNA template from the initial reaction for the internal primer reaction. Thermocycling conditions were as follows: an initial denaturation at 94 °C for 15 min, followed by 25 cycles of denaturation at 94 °C for 1 min, primer annealing at 54 °C (nifH3 & nifH4) and 57°C (nifH1 & nifH2) for 1 min, chain extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplified products (5 ul aliquots) were visualized by agarose gel electrophoresis (1.5% w/v), stained with ethidium bromide, viewed under a UV light source and selected for sequence analysis (approximately 360bp).

Cloning and Sequencing

Purified PCR products were cloned into the plasmid pCR®4-TOPO® (Invitrogen, Carlsbad, CA, USA) and transformed into competent *Escherichia coli* using a TOPO TA Cloning® Kit (Invitrogen) following the manufacturer's protocols. A PCR-based

screening reaction using M13 primers (F: 5' GTA AAA CGA CGG CCA G 3' and R: 5' CAG GAA ACA GCT ATG AC 3') was used to screen for transformed bacterial colonies. Aliquots of 5 ul of all PCR products were analyzed on an agarose gel, and products from clones containing the correct insert size were treated with exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP) (Amersham Biosciences, Piscataway, NJ, USA) prior to sequencing. PCR fragments were bi-directionally sequenced using the Big Dye Terminator kit (Applied Biosystems) with M13 sequencing primers and one-quarter of the recommended concentration of Big Dye. Aliquots of 10 ul of each sequencing reaction product were electrophoretically separated on an ABI 3100 Genetic Analyzer (Applied Biosystems). Fifteen to twenty-one clones were sequenced for each sample.

Sequence Analysis

Sequences were aligned using the MacVector 12.0 DNA sequence analysis software package (Accelerys, San Diego, Calif), trimmed of vector, corrected by manual inspection and analyzed for similarity in BLASTN (Basic Local Alignment Search Tool, NCBI, Bethesda).

Statistical Analysis

Statistical analyses were performed using Statview (SAS Institute). Estuarine sediment NFix data were tested in Statview for the assumptions of ANOVA (normal distribution, equality of variance) using the Kolmogorov–Smirnov Normality Test and the F-test and Bartlett's tests for homogeneity of variance. NFix measurements were log transformed to meet the assumptions of ANOVA. One-way ANOVAs were used to

determine significance differences in sediment characteristics by water depth and region, analyzed individually. One-way ANOVAs were also used to compare NFix rates by water depth alone. Three-way ANOVAs were used to identify significant differences in NFix rates by water depth, region and light/dark incubations and interactions between these factors. Differences were considered significant at $p < 0.05$. Tukey's pair-wise and multiple-comparison tests were used to determine differences between factors from significant ANOVA tests. Residual sum of squares and mean squares were calculated to determine how much of the variance is attributable to random error and the error of the variance, respectively. Simple linear regression analyses were used to examine correlations between mean NFix rates and environmental factors; benthic chl *a*, and sediment NH_4^+. Multiple linear regressions showed no significant improvement in R^2 values.

Results

Sediment Characteristics

Benthic chl *a* measurements ranged from 17 mg m^{-2} to 162 mg m^{-2}. Chl *a* was significantly higher in the shallow (0.5m) sediment, though there was no significant difference between regions (Figure 2A). There was a significant difference in organic content (%) in samples from different regions (Figure 2B, Table 2), with organic content highest in the upper estuarine sediments (mean ± SE, 17.2 % ± 1.4). In addition, there were significant differences between sediment NH_4^+ concentrations based on region and water depth with values ranging from 3.4 mmol N m^{-2} in the shallow lower estuary to 62 mmol N m^{-2} in the deep upper estuary (Figure 2C).

Figure 2: New River Estuary sediment characteristics. (A) mean benthic chl *a* (0-3mm), (B) mean organic matter content (%) (0-10cm), (C) mean sediment extractable NH_4^+ (0-10cm) (mean ± standard error) at three water depths (0.5m, 1.5m, 3.0m) at three sites (Up, Mid, Low) (July 2010).

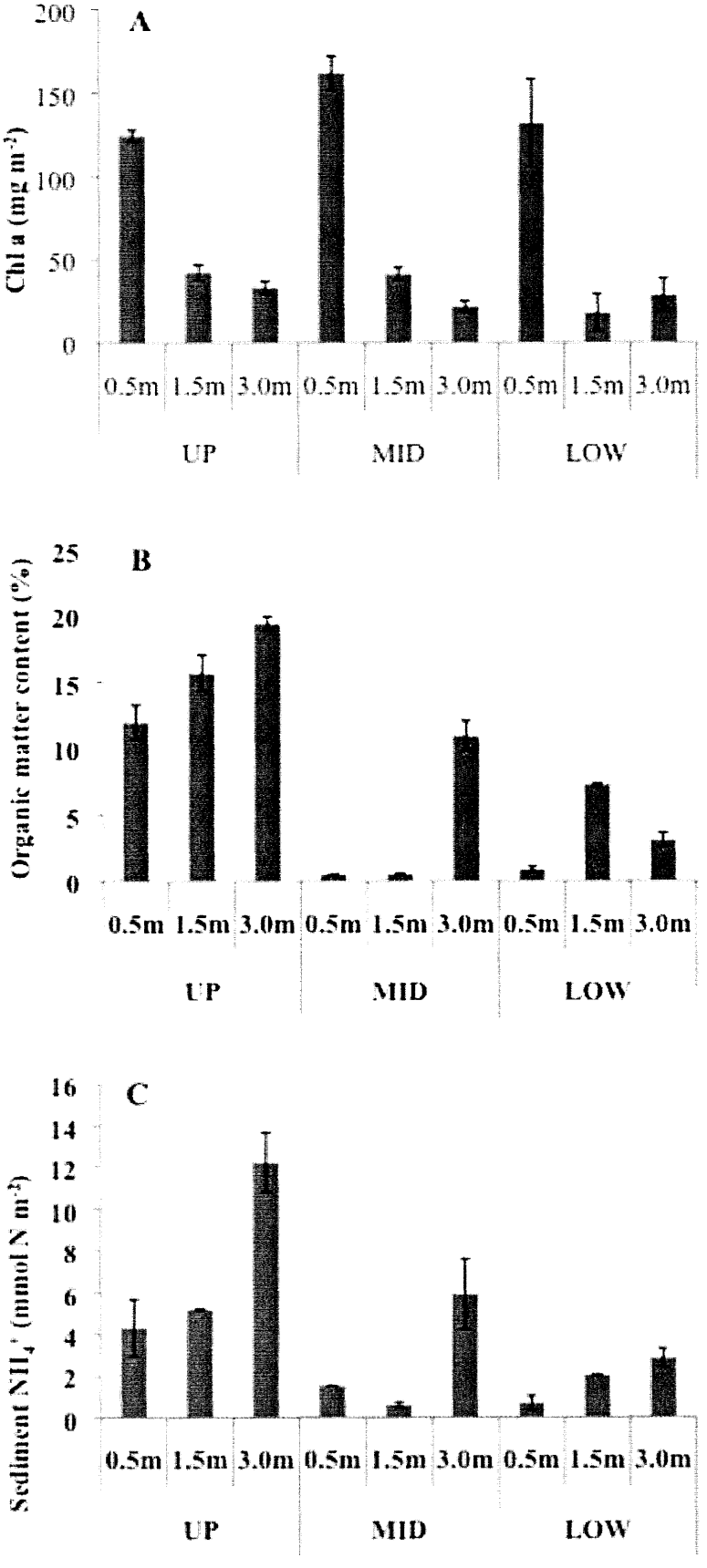

A
Chl a (mg m-3)
200
150
100
50
0
0.5m 1.5m 3.0m 0.5m 1.5m 3.0m 0.5m 1.5m 3.0m
UP
MID
LOW
B
Organic matter content (%)
25
20
15
10
5
0
0.5m 1.5m 3.0m 0.5m 1.5m 3.0m 0.5m 1.5m 3.0m
UP
MID
LOW
C
Sediment NH4+ (mmol N m-2)
16
14
12
10
8
6
4
2
0
0.5m 1.5m 3.0m 0.5m 1.5m 3.0m 0.5m 1.5m 3.0m
UP
MID
LOW

Table 2: Summary of the One-Way ANOVAs of benthic chl *a* (0-3mm), sediment organic matter content (%) (0cm to 10cm Depth Horizon) and sediment NH_4^+ (0-10cm) analyzed individually by water depth and region measured in July 2010. Includes degrees of freedom (DF), Sum of Squares (Sum Sq), Mean Square Variance (Mean Sq), F statistic value (F value), and significant Tukey's pair-wise comparisons ($p < 0.05$), and residual error/ noise (Residuals).

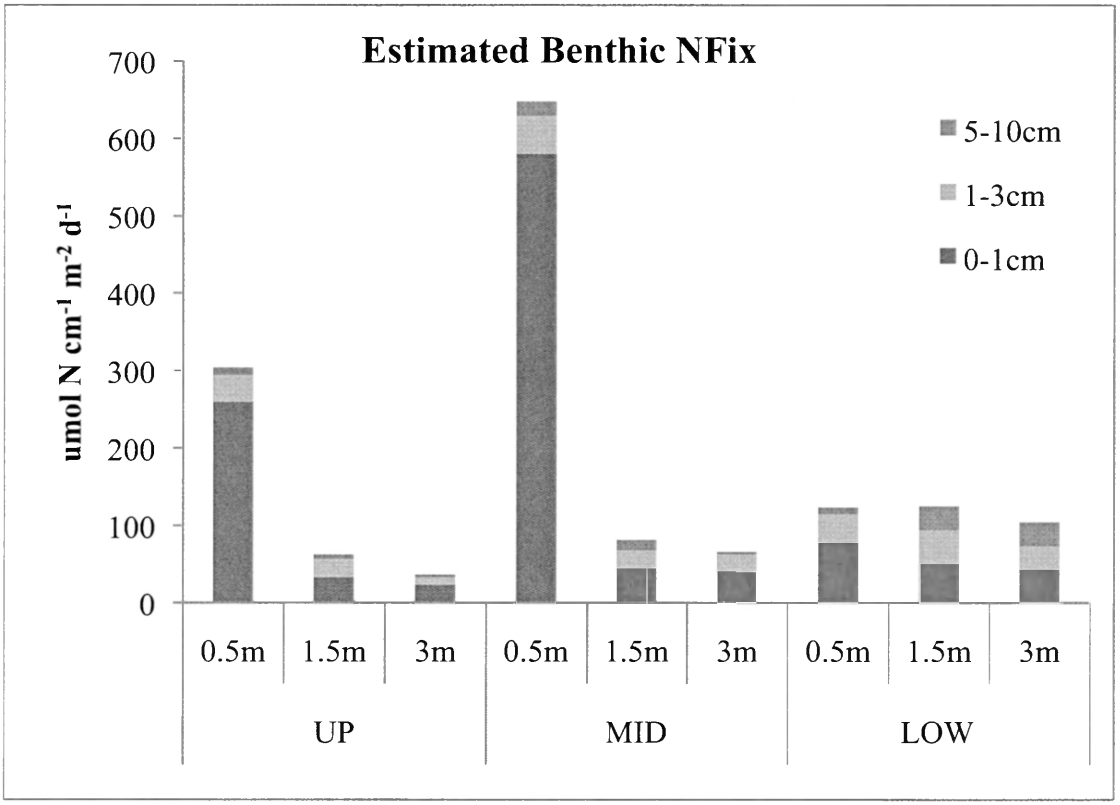
One-Way ANOVAs of Sediment Characteristics						
Parameter	**Df**	**Sum sq**	**Mean Sq**	**F value**	**P Value**	
Chl *a*						
Depth		114138	57070	84.4	**<0.0001**	
Residuals	41	27717	676			
OM%						
Region	2	377.6	188.8	14.6	**0.0049**	Up > (Mid and Low)
Residuals	6	77.4	12.9			
Sed NH_4^+						
Depth	2	140.8	70.4	6.9	**0.0051**	3.0m > (0.5m and 1.5m)
Residuals	21	215	10.3			
Site	2	161.3	80.6	8.7	**0.0018**	Up > (Mid and Low)
Residuals	21	194.8	9.3			

Estuarine-wide NFix measurements

Benthic NFix rates were measured in July 2010, to compare NFix at different water column depths and sediment depths in three regions of the NRE and to relate NFix rates to the distribution of NFixing organisms observed using PCR.(Figure 3). Benthic NFix rates, integrated over 10cm of sediment depth, were significantly higher in shallow

water (0.5m) stations than in deeper water (Table 3). Figure 3 shows that NFix rates were highest in shallow water (0.5 m MSL) at the upper and middle estuary with similar rates for all water depths in the lower region of the estuary.

Figure 3. NFix rates measured at multiple water column depths from three estuarine regions (up, mid, low) in July 2010; rates per cm of sediment depth.



A comparison of NFix through the sediment profile showed that the highest estimated NFix rates per cm of sediment occurred in the 0-1cm section at mid estuary accounting for 69% of the total NFix integrated over the 0-10 cm core (Figure 3). On average, the 1-3 cm and 5-10 cm sections accounted for 25% each of the total 0-10 cm NFix rates. NFix rates integrated over 10cm showed a significant positive correlation

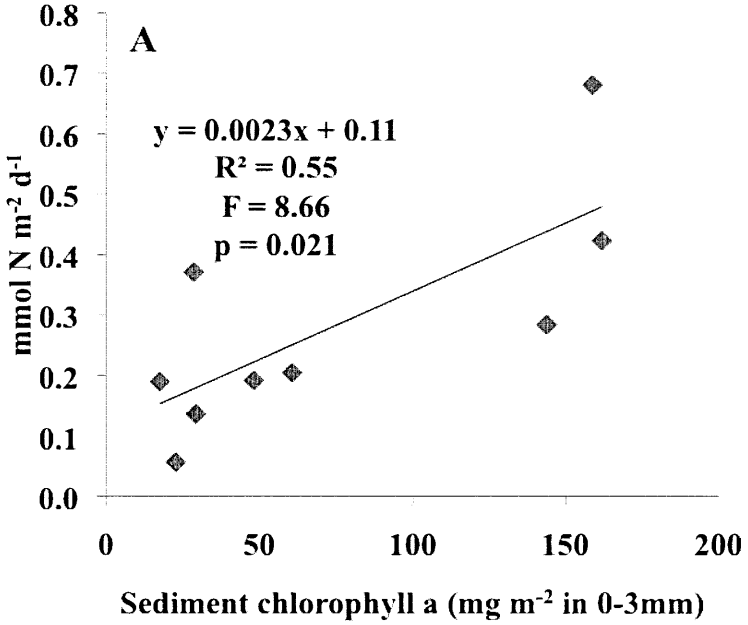
with benthic chl *a* (Figure 4A) and a negative correlation with sediment NH_4^+ concentrations (Figure 4B). There was no significant combined effect of the two environmental factors.

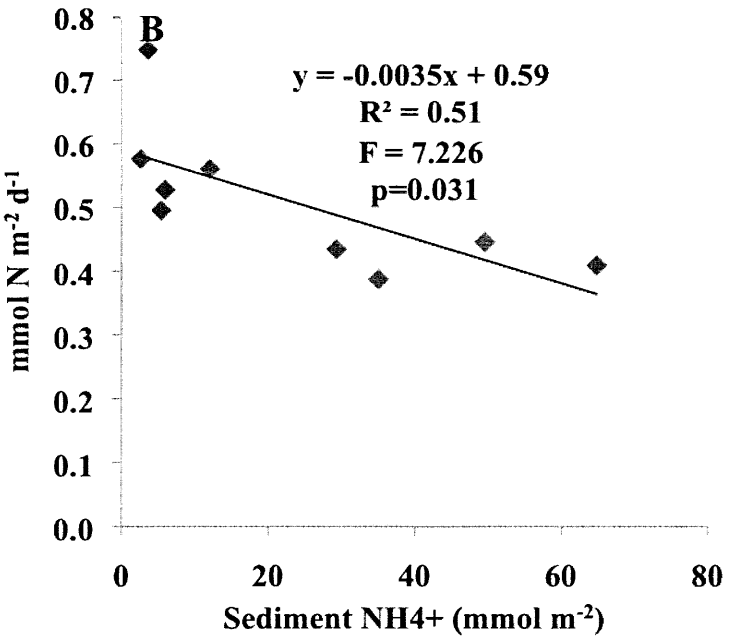
Table 3: Summary of the One-Way ANOVA of sediment NFix rates (0cm to 10cm Depth Horizon) by water depth measured in July 2010. Includes degrees of freedom (DF), Sum of Squares (Sum Sq), Mean Square Variance (Mean Sq), F statistic value (F value), and the significant Tukey's pair-wise comparisons ($p < 0.05$), and residual error/ noise (Residuals).

One-Way ANOVAs of NFix (0cm to 10cm Depth Horizon)

Parameter	**Df**	**Sum sq**	**Mean Sq**	**F value**	**P Value**	
NFix (0-10cm)						
Water depth	2	0.95	0.48	5.1	**0.0147**	0.5m > (1.5 and 3.0m)
Residuals	24	2.3	0.094			

Figure 4. Sediment NFix from July 2010 regressed with (A) benthic chl *a* (mg m^{-2} in 0-3mm) (B) sediment NH_4^+ (0-10cm) (mmol m^{-2}).





NFix rates were significantly higher at the mid estuary site than the upper and lower portions of the system, and there was a significant interaction effect between estuarine region and water depth (Table 4).

Table 4: Three-way ANOVA for NFix (0-1cm Depth Horizon) by Region (Up, Mid, Low), Water depth, and Light/Dark Incubation Measured July 2010. The table provides the parameter evaluated, number of samples (n), the F statistic (region, water depth, light/dark) and degrees of freedom (df; region season, light/dark, error), the probability for each of the main effects (region, season), and the significant Tukey's pair-wise comparisons ($p < 0.05$) for the main effects.

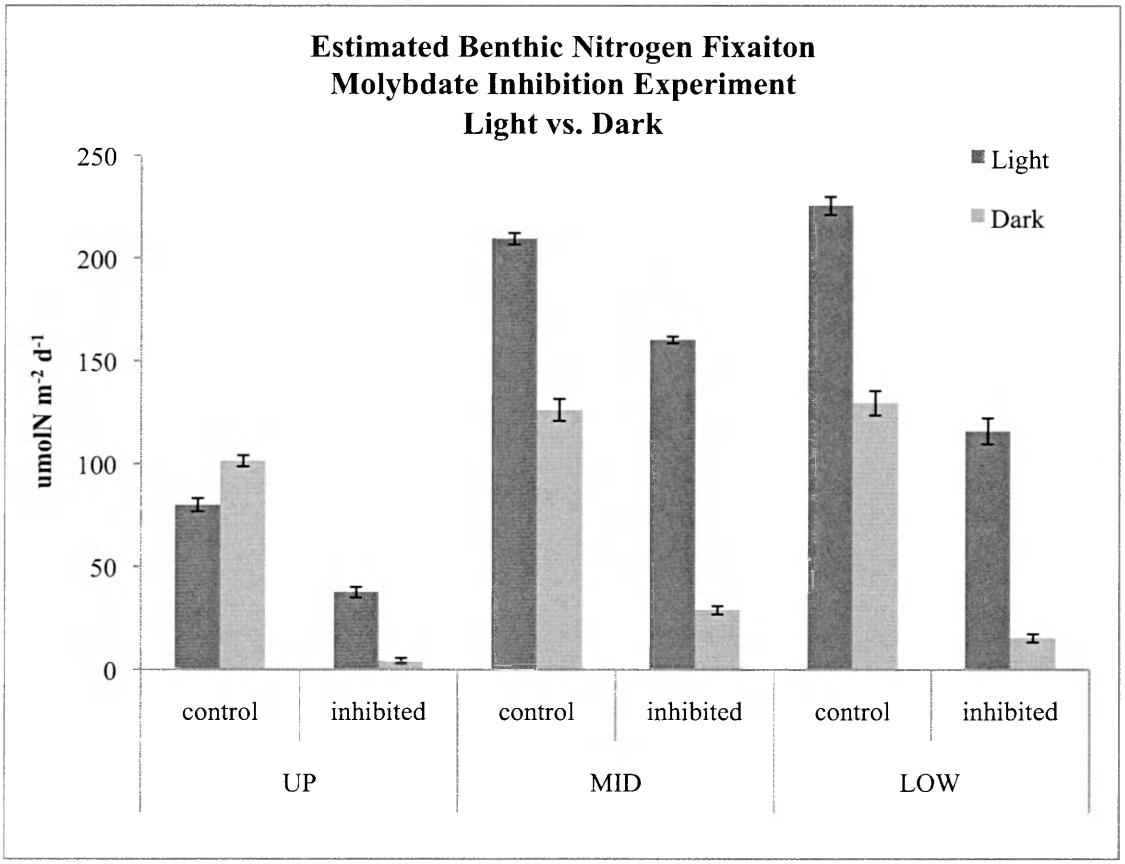
Parameter	n	F	df	Region p-value	Water depth p-value	Light/ Dark p-value	Significant Interaction Effects	Significant Effects
Nfix (0-1cm) Light & Dark	**60**	**18.06, 64.41, 4.51**	**2,1, 1**	**<0.0001**	**<0.0001**	**0.039**	**region * water depth**	**Mid > (Low and Up)** **Light>Dark** **0.5m>1.5m**

To assess the effects of light on NFix rates, we measured rates in light and dark incubations. For the most part we did not observe an effect of light; although surface sediments (0-1cm) collected at the 0.5m water depth exhibited significantly higher benthic NFix rates in the light at the mid site but not at other estuarine sites or water depths in July 2010.

Molybdate inhibition studies were performed on sediments collected in summer 2011 (0.5m water depth) to determine the contribution of sulfate reducing bacteria (SRB) to NFix through the sediment profile (Figure 5 & 6). Molybdate decreased NFix rates by

23-53% in surface sediments (0-1cm) incubated in the light, 77-96% in surface sediments (0-1cm) incubated in the dark. There was no significant difference between NFix rates measured in light and dark in uninhibited samples, but there was a significant difference at all sites between light and dark in samples inhibited with molybdate.

Figure 5. Effect of molybdate inhibition on NFix rates (mean ± standard error) for surface samples (0-1cm) incubated in light and dark. Sediments were collected at the 0.5m water depth in 2011.



SRB contributed a higher percentage to total NFix rates at the upper estuarine site than at the mid estuarine site, consistent with molecular analysis (see below). The percentage contribution through the sediment profile was approximately the same for upper and lower estuarine sites and highest in the 1-3cm sediment depth section for the mid site.

A one-way ANOVA was conducted to test the effect of molybdate inhibition on NFix rates for each sediment depth at all sites (Table 5). Molybdate significantly inhibited NFix at all but one site and sediment depth (Mid 1-3cm) (Figure 6); they were responsible for 44-83% of NFix observed in the New River Estuary.

Figure 6. Effect of molybdate inhibition on NFix rates (mean ± standard error) in sections through the sediment profile. Sediments were collected at the 0.5m water depth in 2011.

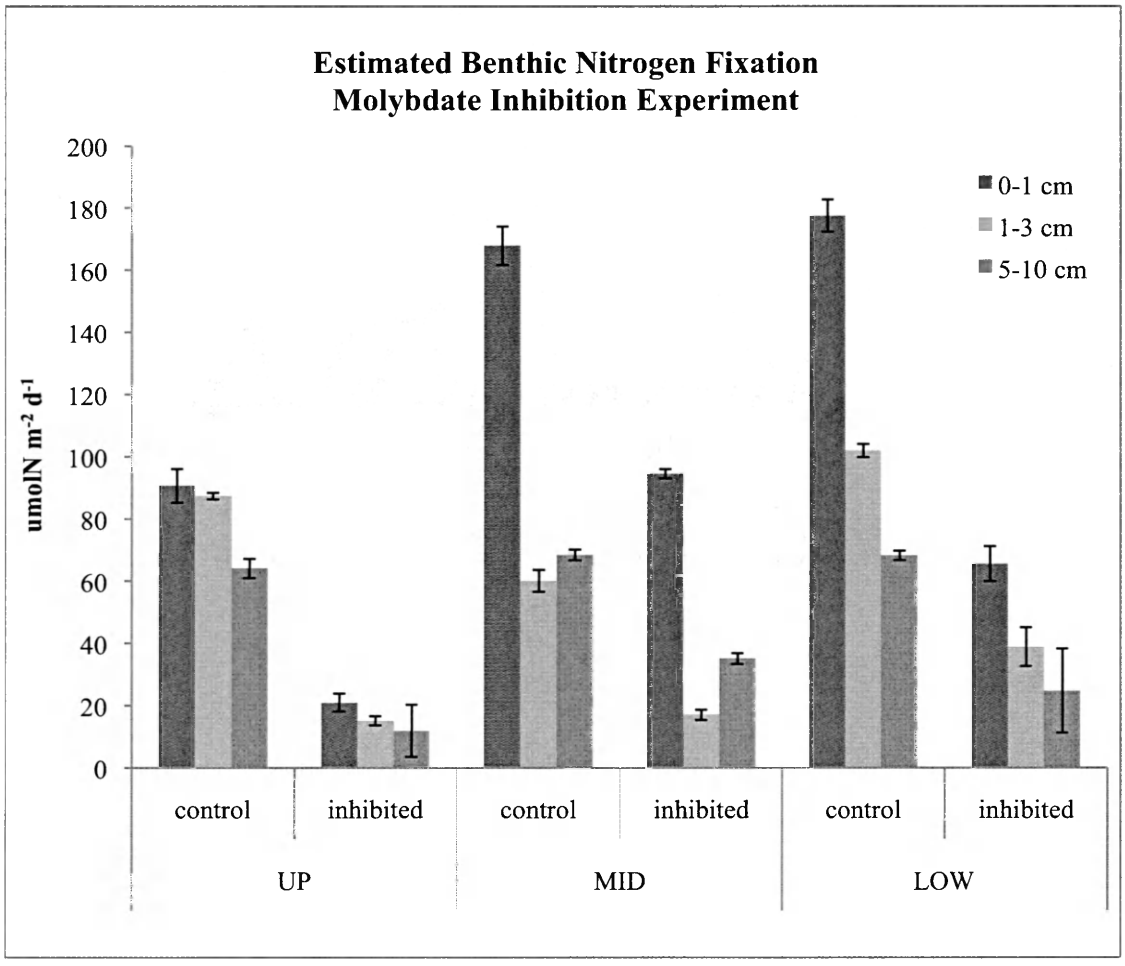


Table 5: One-way ANOVA results for molybdate inhibition experiment. This table provides the mean + SE NFix rate value for both inhibited and uninhibited samples, their percent contribution to total rates integrated over 10cm, and significant Tukey's pair-wise comparisons ($p < 0.05$) between inhibited and uninhibited values.

	Control		+20 mM molybdate			
Site	Mean	SE	Mean	SE	% contribution of SRB	One-way Anova $\alpha = 0.05$
UP						
0-1 cm	91	5.3	21	2.9	77	$p = 0.001$
1-3 cm	87	1.1	15	1.5	83	$p < 0.001$
5-10 cm	64	3.1	12	8.3	81	*$p = 0.0323$*
MID						
0-1 cm	170	6.2	95	1.4	44	$p = 0.0388$
1-3 cm	60	3.5	17	1.6	72	$p = 0.0677$
5-10 cm	68	1.7	35	1.8	49	$p = 0.0310$
LOW						
0-1 cm	180	5.1	66	5.6	63	$p = 0.0027$
1-3 cm	100	2.1	39	6.2	62	$p = 0.0333$
5-10 cm	68	1.6	25	13	64	$p = 0.0245$

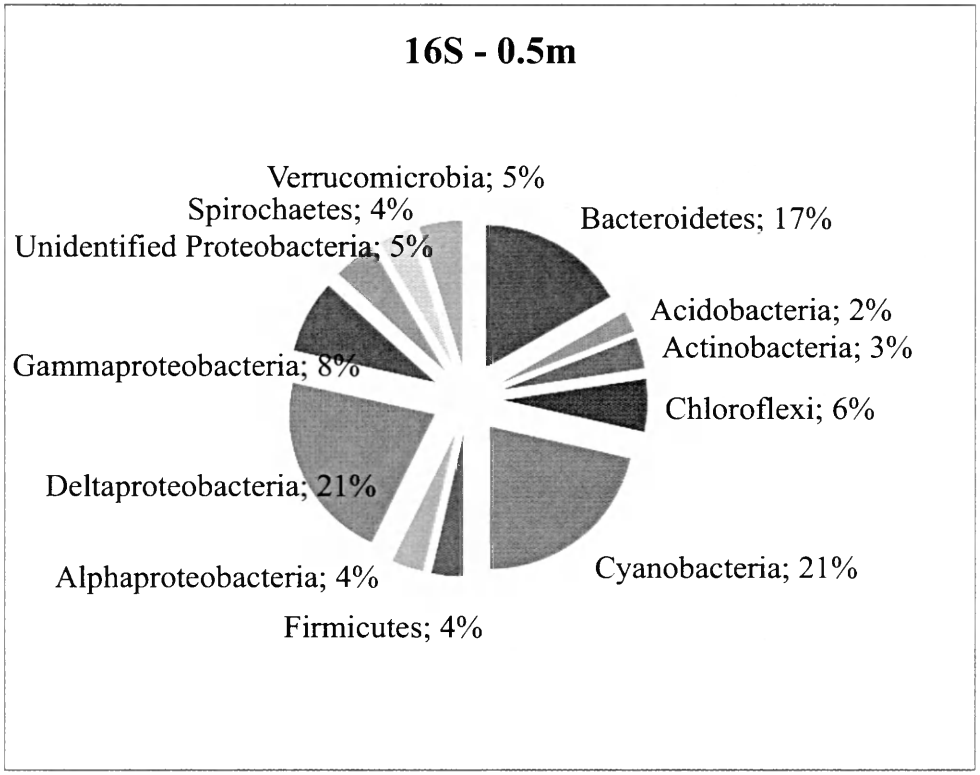
16S and *NifH* molecular analysis

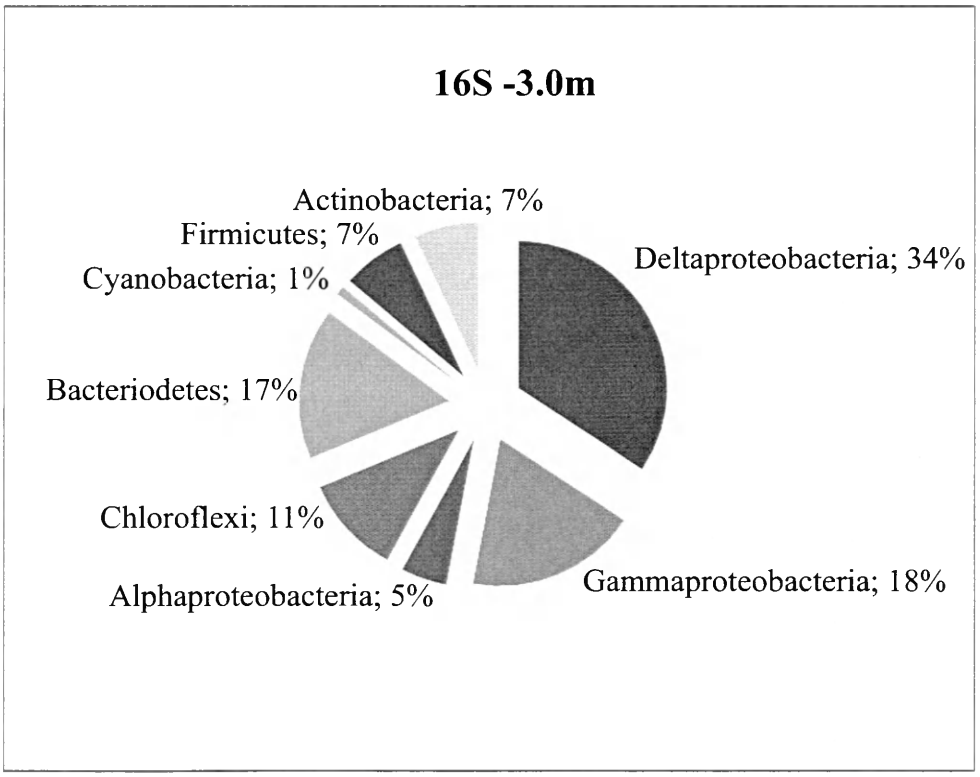
To determine the diversity and distribution of bacteria in sediments of the NRE we used PCR to amplify 16S ribosomal DNA gene sequences on samples taken in July 2010 from the upper, middle, and lower estuary regions at 0.5m and 3.0m water depths. A total of 194 16S (Figure 7) sequences were analyzed. Then to identify the potential NFixers in NRE sediments we used PCR to amplify *nifH* gene sequences from the same

sediment samples taken in July 2010. A total of 188 *nifH* sequences were analyzed (Figure 8).

The majority of the bacterial groups identified using the 16S gene were similar for both shallow and deep water sediments, though as expected cyanobacteria made up a much greater percentage of the sequences in the shallow water sediments (Appendix 1). The majority of the sequences in both the shallow and deep water sediments were identified as uncultured and could only be classified by bacterial family. In the shallow water samples, the only species specifically identified included *Pseudomonas* sp., *Mesoflavibacter zeaxanthinifaciens*, *Synechococcus* sp., and *Pseudanabaena* sp. The species identified in the deep water sediments included *Thiorhodospira* sp., *Fusobacterium* sp., *Halia* sp. in the upper estuary, and *Thioalkaivibrio versutus* and *Algibacter* in the lower estuary.

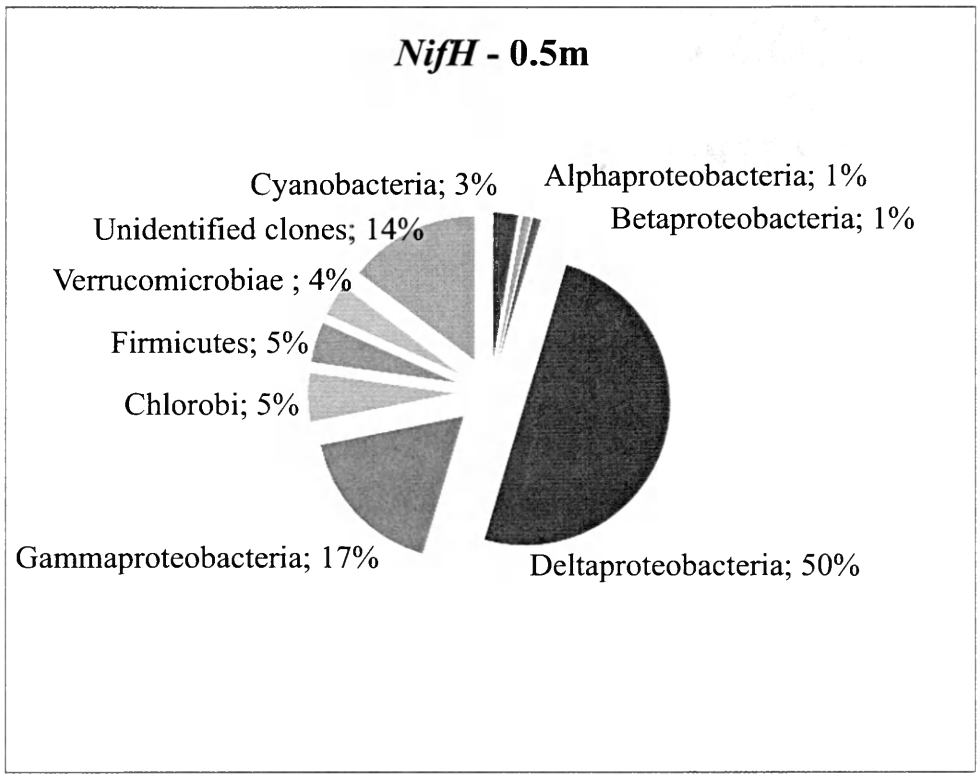
Figure 7: General microbial community composition determined by 16S PCR results for sediment samples collected in July 2009 (0-1cm & 1-3cm sediment section). Bacterial groups were identified using BLASTN (Basic Local Alignment Search Tool, NCBI, Bethesda).

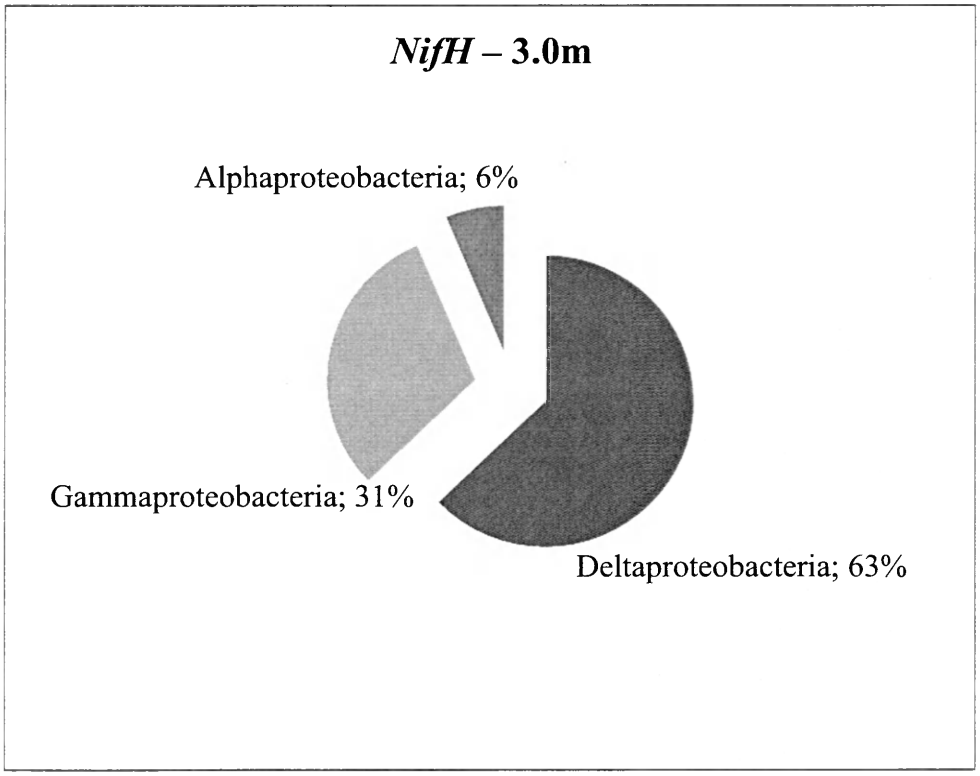

16S - 0.5m
Verrucomicrobia; 5%
Spirochaetes; 4%
Unidentified Proteobacteria; 5%
Bacteroidetes; 17%
Acidobacteria; 2%
Actinobacteria; 3%
Chloroflexi; 6%
Gammaproteobacteria; 8%
Deltaproteobacteria; 21%
Alphaproteobacteria; 4%
Cyanobacteria; 21%
Firmicutes; 4%


16S -3.0m
Actinobacteria; 7%
Firmicutes; 7%
Deltaproteobacteria; 34%
Cyanobacteria; 1%
Bacteriodetes; 17%
Chloroflexi; 11%
Alphaproteobacteria; 5%
Gammaproteobacteria; 18%

Cyanobacterial *nifH* genes, identified in the surface sediments at shallow water depths, were all shown to be related to *Microcoleus chthonoplastes*. In the majority of the deeper water samples, as well as in many of the shallow samples, the *nifH* genes that were detected were most closely related to SRB's known to fix N, including *Desulfovibrio* spp. and *Desulfobacter* spp. Twenty-one of the *nifH* sequences identified in the shallow water depth in the mid and lower estuary were related *to Desulfatibacillum alkenivorans*; twenty throughout the estuary were related to *Desulfovibrio desulfuricans*. There were nine sequences related to *Ectothiorhodospira mobilis*, a phototrophic purple sulfur bacteria (Tourova et al. 2007), found mostly in the mid estuary. In the deep water (3m) sediments, the largest number of sequences (32) were related to *Desulfovibrio desulfuricans*. There were nine sequences related to *Ectothiorhodospira mobili*, and twelve related to *Halorhodospira halophila*, also a phototrophic purple sulfur bacteria in the family *Ectothiorhodospiraceae*. The remainder of the deep water sediment sequences were related to a variety of delta proteobacteria including *Desulfovibrio vulgaris* and *Pelobacter carbinolicus*, an uncultured alphaproteobacteria. The sequences identified in the 3m water depth samples were found throughout the estuary except the alphaproteobacteria, which were found only in the lower estuary.

Figure 8: *NifH* PCR results for sediment samples collected in July 2009 (0-1cm & 1-3cm sediment section). Bacterial groups were identified using BLASTN (Basic Local Alignment Search Tool, NCBI, Bethesda).


NifH - 0.5m
Cyanobacteria; 3%
Alphaproteobacteria; 1%
Betaproteobacteria; 1%
Unidentified clones; 14%
Verrucomicrobiae ; 4%
Firmicutes; 5%
Chlorobi; 5%
Gammaproteobacteria; 17%
Deltaproteobacteria; 50%


NifH – 3.0m
Alphaproteobacteria; 6%
Gammaproteobacteria; 31%
Deltaproteobacteria; 63%

Discussion

In most estuarine systems pelagic N-fixing organisms are thought to be scarce or absent, though a diverse group of N-fixers have been detected in the water column of the Chesapeake Bay (Short et al. 2004). In contrast, in oligotrophic tropical and subtropical ocean waters, the greatest source of fixed N is thought to be NFix, with a substantial amount contributed by *Trichodesmium* alone (Capone et al. 1997, Capone et al. 2005).

NFix has not generally been considered an important source of biologically available N in most coastal systems, with the majority of studies focusing on coral reef, microbial mat, and macrophyte communities, where it has been shown to be an important source of N(McCarthy 1980, Howarth and Marino 2006, Carpenter and Capone 2008). Although estuarine sediments are generally thought to exhibit low rates of NFix activity, organisms that potentially can fix nitrogen have been identified in a number of estuaries (Burns 2002, Short 2005, Bertics 2010); however, the relationship between activity and presence of *nifH* genes is uncertain since Burns et al (2002) did not find a correlation between the presence of *nifH* genes and NFix activity in the oligohaline Neuse River estuary, NC. In recent studies high rates of NFix have been identified in some deep estuarine sediments (Fulweiler et al. 2007), eelgrass dominated shallow coastal sediments (McGlathery 1998, Cole and McGlathery 2012), and shallow sediments with and without microbial mat populations (Bertics et al. 2010, Steppe and Paerl 2005) suggesting it may

be more important than previously thought. A comparison between rates found in our study of the NRE and these other studies is shown in Table 6.

Table 6: Integrated NFix rates in the NRE sediments (July 2010) compared to other sites with different sediment types.

Location	Depth of integration (cm)	umol N m^{-2} d^{-1} (using 4:1 ratio C_2H_2:N_2, which corresponds to 2:1 C_2H_4:NH_3)
Eelgrass rhizosphere (McGlathery et al. 1998)	0 to 14	71 to 430
Eelgrass meadow sediments (Cole and McGlathery 2012)	0 to 5	146 to 390
Subtidal bioturbated sediments (Bertics et al 2010)	1 to 10	150 to 2430
Subtidal bioturbated sediments – with microbial mat (Bertics et al 2010)	0 to 10	160 to 8050
Subtidal sediments to 25cm depth (~28m water depth) (Bertics et al. 2012)	0 to 25	80 to 220
Intertidal lagoon sediments (Charpy-Roubaud et al. 2001)	0 to 2	10 to 570
Subtidal sediments (~8m water depth) (Fulweiler et al. 2007)	0 to 30.5	600 - 15,600
Intertidal microbial mat (Steppe & Paerl 2005)	0 to 5	1630 ± 1150
New River Estuary subtidal sediments (0.5m to 3m water depth) (this study)	0 to 10	56 to 950

Cyanobacteria, thought of as the major NFixers in fresh and oceanic waters (Zehr 2003) have been shown to contribute to high rates of NFix in shallow and intertidal microbial mats on a sandflat near Beaufort, NC (Steppe & Paerl 2005) and on a Dutch

barrier island (Severin and Stal 2010). In the NRE; however, our results suggested that cyanobacteria are not likely major contributors to NFix in the benthos due to the low number of cyanobacterial *nifH* sequences identified and the results of molybdate inhibition, which indicated that SRB were responsible for 44 – 83% of observed NFix activity. Other bacterial autotrophs, which may contribute to NFix in the NRE are the, purple sulfur bacteria, examples of which were identified throughout the estuary. However, we recognize that since we did not measure *nifH* gene expression or abundance, the presence of a bacterial group with *nifH* provides only a hint that it may contribute to observed NFix. The capacity for NFix has been shown for most purple phototrophic bacteria, including some members of the *Ectothiorhodospiraceae* , such as *Ectothiorhodospir* and, *Halorhodospira* (Tourova et al. 2007), which have been identified in this study as well as previously in microbial mats (Severin and Stal 2010).

In out study, we found that benthic NFix was generally not significantly higher in light than in dark incubations; only significantly higher in light incubations at the mid estuary site at 0.5m water depth. We also found that *nifH* containing cyanobacteria did not appear to be common; therefore we suggest that non-cyanobacterial phototrophs are likely responsible for much of the light-dependent NFix observed in he NRE. However, our results suggest that heterotrophic sulfate reducing bacteria are the dominant NFixers in this shallow photic estuary.

The high sulfate concentrations often found in estuarine sediments can slow the growth rate of cyanobacteria, making the sediments a more favorable environment for other NFixing organisms that use sulfate as an energy source (Howarth et al. 1988a, Marino et al. 2003). In the present study, the presence of molybdate decreased NFix

activity by 23-53% in surface sediments (0-1cm) incubated in the light, 77-96% in surface sediments (0-1cm) incubated in the dark (Figure 5), and 49-83% in deeper sediments (1-3cm & 5-10cm) (Figure 6 & Table 5). Similar results were found in sediments up to 10cm deep collected in Catalina Harbor, CA where NFix rates were inhibited by at least 74.7 ± 6% (Bertics et al 2010). NFix has also been shown to be coupled to sulfate reduction in deep water sediment (~28m) up to 25cm deep (Bertics et al 2012). Among the potential heterotrophic NFixers identified in the NRE, *Desulfovibrio vulgaris* was also isolated in sediments below deep hypoxic waters in Eckernförde Bay, Baltic Sea (Bertics et al 2012), in salt marsh rhizoshperes (Davis et al. 2011), in microbial mats (Moisander et al 2006), and in Chesapeake Bay sediments (Moisander et al 2007).

Desulfovibrio desulfuricans, which has also been shown to perform DNRA as well as fix N, (Keith and Herbert 1983) was identified in NRE sediments and has also been identified in the sandy marine sediments of Santa Rosa Sound, FL (Devereux and Mundfrom 1994). *Pelobacter carbinolicus,* an iron and sulfate reducer, (Lovely 1995) was identified in this study as well as previously in Catalina Harbor sediments (Bertics et al. 2010). The single NFixing cyanobacteria found in this study, *Microcoleus chthonoplastes,* a filamentous non-heterocystous cyanobacteria, has been isolated in many marine environments and was the dominant cyanobacterial component observed in previous studies on microbial mats (Severin and Stal 2010) and salt marsh sediments (Currin and Paerl 1996). Although the NFixing capability of *Microcoleus chthonoplastes* has been questioned, it was identified with the *nifH* gene in this study and the complete *nif*-gene cluster has been shown to be present in the genome of *M. chthonoplastes* PCC 7420 (Bolhuis et al 2010).

A diverse assemblage of potential NFixers were identified in the NRE sediments, as well as in other studies of coastal temperate estuaries including the Neuse River Estuary and the Chesapeake Bay (Burns 2002, Short 2004). The results of this study suggest that SRB's are important contributors to NFix rates throughout the estuary and at all water depths, though further studies are required to relate the presence of these organisms with activity.

Conclusion

To assess the distribution of benthic N-fixation as a function of light and depth, we measured benthic NFix rates along the estuarine gradient at multiple water depths (with a range of light availabilities) and multiple sediment depths. Although highest rates of N-fixation were in the top 0 – 1 cm shallow sediments, a substantial portion occurred down to 10 cm in the shallow and deeper water sediments. N-fixation showed a negative relationship to ambient ammonium levels, and a positive relationship with benthic chl *a*. We incubated surface sediments in both light and dark and used molybdate inhibition as well as PCR to determine the relative contributions of autotrophic and heterotrophic N-fixers to the benthos. A wide variety of diazotrophs were detected using PCR, the majority being sulfate-reducing bacteria. The importance of sulfate reducing bacteria to benthic NFix in the NRE was supported by results showing 44-83% of NFix being inhibited by molybdate addition. The possibility that phototrophic non-cyanobacteria are important NFixers in the NRE was supported by PCR results, though the majority of NFix was dependent on sulfate reduction.

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Appendix 1: PCR results for 16S and *nifH* analysis. Results are listed as number of sequences identified within each bacterial group by estuary region and water depth.

16S Results						
Water Depth	0.5m			3m		
Estuary Region	UP	MID	LOW	UP	MID	LOW
Acidobacteria		1	1			
Actinobacteria	2	1		1	3	3
Alphaproteobacteria		1	2	3	1	1
Bacterial Group						
Bacteriodetes	4	5	5	4	4	9
Betaproteobacteria					1	
Chloroflexi	5			2	4	5
Cyanobacteria	3	7	8	1		
Deferribacteres	2				1	
Deltaproteobacteria	4	8	6	10	13	12
Firmicutes		1	2	2	3	2
Fusobacteria				1		
Gammaproteobacteria	4	1	2	6	7	6
Planctomycetes						1
Proteobacteria		1	3			
Spirochaetes		2	1	1		
Verrucomicrobia	2	1	1	1		
***NifH* Results**						
Water Depth	0.5m			3m		
Estuary Region	UP	MID	LOW	UP	MID	LOW
Alphaproteobacteria		1				5
Betaproteobacteria	1					
Chlorobi		4	2			
Cyanobacteria	1	2				
Deltaproteobacteria	15	16	24	15	16	18
Firmicutes	4	1				
Gammaproteobacteria	2	13	4	9	7	8
Unidentified clones	9	2	5			
Verrucomicrobiae	3	1				

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